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University of Alberta

Characterization of a Cold Shock Cyanobacterial RNA Helicase Protein

by

Esther Huiting Yu 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Microbiology and Biotechnology

Department of Biological Sciences

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Characterization of a Cold Shock Cyanobacterial RNA Helicase Protein** submitted by **Esther Huiting Yu** in partial fulfillment of the requirements for the degree of **Master of Science** in **Microbiology and Biotechnology**.

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Dr. Roseline Godbout

January 14, 1999
Date

DEDICATION

To my mother, Cuiduan, and my sister, Huiwen, who have supported me in the completion of this study

ABSTRACT

A putative RNA helicase gene, designated *crhC* (cyanobacterial RNA helicase-like Cold) was overexpressed in *E. coli* and characterized both biochemically and immunologically. Overexpression of this protein was obtained using two systems: pGEX and pRSET systems. Soluble CrhC protein could be produced by both systems when the overexpression was carried out at 20°C. However, a large amount of purified, soluble CrhC could only be obtained from the pRSET system. Purified CrhC protein was used to produce polyclonal antibodies and conduct biochemical enzyme assays. Western analyses indicate that the protein is expressed only as a result of cold shock. Sequence analyses of the 5' untranslated region of *crhC* gene suggest that *crhC* possesses cold shock regulatory elements similar to that of *E. coli* cold shock genes. Native protein gel analyses and immunoprecipitations suggest the association of CrhC protein with a multi-subunit complex. This hypothesis was further tested by Far-Western analyses, which indicates a direct interaction between CrhC and a 37 kDa protein. Biochemical enzyme assays show that CrhC possesses RNA-dependent ATPase, ATP-independent RNA binding, and ATP-dependent RNA unwinding activities, thus establishing CrhC as an RNA helicase. The results and the possible role of CrhC in the cold shock response in *Anabaena* sp. PCC 7120 are discussed.

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APS	ammonium persulfate
APT	adenosine 5'-triphosphate
bp	basepair(s)
BSA	bovine serum albumin
°C	degrees centigrade
cpm	counts per minute
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	any deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
g	gram(s)
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
h	hour(s)
IPTG	isopropyl - β - D - thiogalactoside
kb	kilobase(s)/kilobasepair(s)
kDa	kiloDalton(s)
L	liter(s)
M	molar
m^2	meter(s) square
mg	milligram(s)
min	minute(s)

mL	milliliter(s)
mM	millimolar
MOPS	3-[N-Morpholino]propanesulfonic acid
nm	nanometer(s)
ng	nanogram(s)
OD	optical density
PMSF	phenylmethylsulfonyl fluoride
%	percent
pmol	picomole(s)
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
sarkosyl	N-laurylsarcosine
SDS	sodium dodecyl sulfate
sec	second(s)
TAE	Tris/acetate/EDTA
TBE	Tris/borate/EDTA
TBS	Tris buffered saline
TEMED	N,N,N',N' - tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
µg	microgram(s)
µL	microliter(s)
µM	micromolar

UV	ultraviolet
V	volt(s)
v/v	volume per volume
W	watt(s)
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER ONE
INTRODUCTION

1.1 RNA HELICASE

1.1.1 General Characteristics of RNA Helicases

RNA molecules play an essential role in many cellular processes, often as components of ribonucleoprotein complexes. Like proteins, RNA molecules adopt sequence-specific secondary and tertiary structures that are essential for function; alteration of these structures therefore provides a means of regulating RNA function (Fuller-Pace, 1994). RNA helicases are a group of proteins that can modulate RNA structure through their ability to unwind RNA duplexed regions using the energy obtained from ATP hydrolysis.

RNA helicases, originally known as "DEAD" box proteins due to their conserved Asp-Glu-Ala-Asp (DEAD in single-letter code) motif, are actually a large family of proteins that have several subgroups including the DEAD, DEAH and DEXH subgroups (Fuller-Pace and Lane, 1992). It is also evident that this family forms part of a larger group of proteins that interact with polynucleotides and nucleoside triphosphates (Koonin, 1991; Gorbalenya *et al.*, 1989) and the larger group of proteins are divided into three superfamilies and two smaller families (Gorbalenya and Koonin, 1993). RNA helicases belong to superfamily SF2, which also include a number of DNA repair, recombination and replication enzymes. They are identified according to their amino acid sequence similarity. As shown in Figure 1.1, each subgroup of proteins share a common core region of 300-350 amino acids that consists of six to eight conserved motifs that are thought to be essential for their enzymatic activities. The amino acid spacing between each conserved motifs is conserved, but sequence in these areas is not. In addition to this core region, individual family members have distinct amino and carboxy-terminal regions that vary in length. These regions, as well as differences within the core region, may either determine the

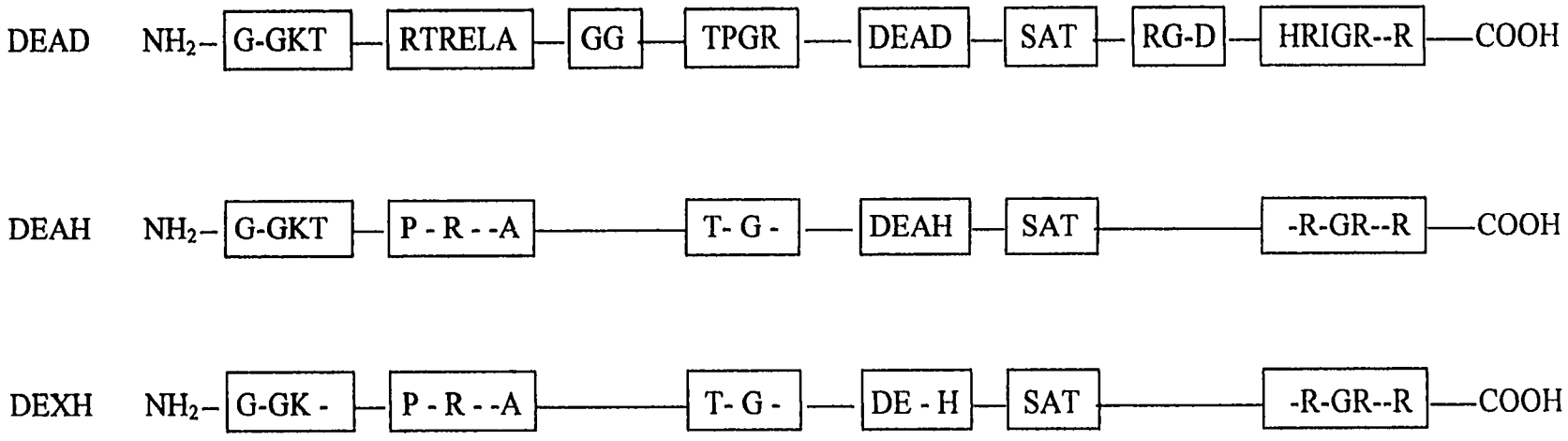


Figure 1.1 Schematic representations of the DEAD, DEAH and DEXH box RNA helicase families. Boxed regions indicate conserved amino acid motifs. Diagrams are not to scale. (Fuller-Pace, 1994)

substrate specificity, or bind accessory proteins. As more proteins are assigned to the superfamily, it is clear that it includes DNA helicases as well as RNA helicases, and perhaps even translocases (Koonin, 1991; Fuller-Pace, 1994). Therefore, in addition to the criterion of sequence similarity, biochemical activity should also be considered in defining members of the family.

1.1.2 *in vitro* Enzymatic Activities

Theoretically, RNA helicases should possess RNA-dependent ATPase activity and ATP-dependent RNA unwinding activity. *In vitro* enzymatic assays have been carried out extensively; however, only a few RNA helicases were reported to have both activities. These proteins include: the prototype RNA helicase eIF-4A (*e*ukaryotic translation *i*nitiation *f*actor *4A*) (Grifo *et al.*, 1984; Rozen *et al.*, 1990), human p68 protein (Hirling *et al.*, 1989), vaccinia virus DEXH protein NPH-2 (Shuman, 1992), plum pox virus DEAH protein CI (Lain *et al.*, 1990), hepatitis C virus NS3 protein (Gallinari *et al.*, 1998), Dbp5 (Tseng *et al.*, 1998), Prp16, Prp22 (Wang *et al.*, 1998), and Upf1 from *Saccharomyces cerevisiae* (Weng *et al.*, 1998), and the AtDRH1 protein from *Arabidopsis thaliana* (Okanami *et al*, 1998).

A number of RNA helicases have been shown to possess RNA-dependent ATPase activity but not unwinding activity, such as a number of PRP proteins from *S. cerevisiae* that are involved in RNA splicing (Schwer and Guthrie, 1991; Kim *et al.*, 1992), the Rrp3 protein from *S. cerevisiae* that is required for 18S rRNA processing (O'Day *et al.*, 1996), and the Rubella virus RNA helicase whose sequence is localized in the nonstructural polyprotein sequence (Gros and Wengler, 1996). The ATPase activities of some RNA helicases are not RNA-dependent; for example, the An3 protein from *Xenopus laevis* (Gururajan and Weeks, 1997). Furthermore, some RNA helicases have ATP-independent RNA destabilizing activity. The best example is the *Escherichia coli* DEAD box protein A (DbpA) (Boddeker *et al.*, 1997). Boddeker *et al.* proposed that the ATP-independent RNA destabilizing activity of DbpA is due to its unusual 70 amino acid C-terminal domain. It

was shown that 25% of its C-terminal domain consists of positively charged residues and 29% are small (Gly/Ala) residues. Therefore, it is possible that the C-terminal region of the protein is responsible for RNA binding and destabilizing. In addition to the above examples, many RNA helicases have been shown to hydrolyze not only ATP but also other nucleoside triphosphates in an RNA-dependent fashion (Gallinari *et al.*, 1998; Weng *et al.*, 1998; Lain *et al.*, 1990; Shuman, 1992).

Aside from the helicases mentioned above, a large number of RNA helicases have not yet been tested for enzymatic activities *in vitro*, and thus remain as putative RNA helicases.

1.1.3 Functions of the Conserved Motifs

In vitro enzymatic reactions have been performed not only to determine the activities of various RNA helicases, but also to elucidate the functions of the conserved motifs. Extensive mutational analysis of the conserved domains in eIF-4A to assess their role in ATP hydrolysis and RNA unwinding has been carried out by several groups. Schmid and Linder (1991) carried out *in vivo* viability tests; Pause and Sonenberg (1992 and 1993) performed *in vitro* enzymatic assays. Motif AXXGXGKT is typical of the A motif of ATP-binding proteins (Walker, 1982). Linder *et al.* (1989) demonstrated that mutations of the alanine residue to valine or aspartic acid decreased growth and *in vivo* translation rates. Rozen *et al.* (1989) showed that substitution of the lysine residue with asparagine eliminated ATP binding activity. Pause and Sonenberg (1992) concluded that this motif is required for ATP binding and that the lysine residue is particularly important in this binding. These conclusions are not surprising because the lysine residue has been shown to bind the β– and γ– phosphates of ATP (Pause and Sonenberg, 1993).

The DEAD motif represents a modified Walker B motif of ATP-binding proteins (Walker *et al.*, 1982). It is suggested that this motif is critical for ATP hydrolysis, but it is not essential for ATP binding, because a mutation in any of the four positions did not

interfere with this activity (Pause and Sonenberg 1993; Magee, 1997). Substitution of the second aspartic acid residue with histidine resulted in a three-fold elevation in ATPase activity but a 10-fold reduction in RNA helicase activity suggesting that this motif is important in coupling ATPase and RNA unwinding activities.

The third motif, the SAT box, which is unique to the helicase superfamily II, a group which also includes a number of DNA repair, recombination and replication enzymes (Gorbalenya and Koonin, 1993), was studied by Pause and Sonenberg (1992). They found that mutations at both the serine and threonine residues did not affect ATP binding and ATPase activities while the RNA unwinding activity was abrogated by the mutations. Plumpton *et al.* (1994) showed that mutations in the SAT motif of PRP2 reduce ATP hydrolysis and completely abolish splicing with PRP2 remaining associated with stalled spliceosome complexes. These investigations suggest that the SAT motif is required for RNA unwinding and may also couple ATP hydrolysis to RNA unwinding. Although the SAT motif is believed to be conserved in RNA helicases, alteration of this motif has been found in some of the members. For instance, several viral helicases, which belong to the DEXH family, have a TAT box instead of the SAT box. The RNA helicase in this study, CrhC, isolated from *Anabaena* sp. strain PCC 7120 also has an altered SAT motif, a FAT motif (Magee, 1997). The alteration of the SAT to TAT or FAT may therefore provide some functional specificity related to RNA unwinding for the RNA helicases.

The only other motif that has been examined is the HRIGRXXR box. Schmid and Linder (1991) demonstrated that a mutation in the first arginine (R) resulted in non-viable cells except when lysine was substituted, thus suggesting that a positive charge is necessary at this position. Pause and Sonenberg (1993) reported that any mutation, conservative or not, in any one of the three arginines in the HRIGRXXR sequence drastically reduce eIF-4A binding to RNA and abrogate RNA helicase activity. Some of these mutations also affected ATP binding and ATPase activity. They concluded that the HRIGRXXR region is involved in the ATP hydrolysis reaction and the coupling of ATPase and RNA-binding/helicase activities.

So far, only four out of the eight conserved motifs have been studied. The functions of the remaining motifs, PTRELA, GG, TPGR and GRXD have not been determined. However, their conservation suggests that they are somehow involved in the ATP binding, ATP hydrolysis and/or RNA unwinding activities.

1.1.4 RNA Helicases Are Involved in a Wide Range of Cellular Processes

RNA helicases are an essential component of many fundamental cellular processes including translation initiation, ribosome assembly and mRNA splicing. Among all the helicases that have been characterized, a large number of them are encoded in dsRNA viral genome where they are believed to be involved in genome replication (Gross and Shuman, 1998; van der Meer *et al.*, 1998; Fuller-Pace, 1994).

eIF-4A (eukaryotic translation initiation factor 4A) is the best-characterized DEAD box protein that has been found in various mammals and plants. It has been shown to have RNA-dependent ATPase activity and ATP-dependent mRNA unwinding activity (Grifo *et al.*, 1984). The importance of eIF-4A lies in its central role in translation initiation where it unwinds mRNA secondary structure in the 5' untranslated region in order to allow binding of the 40S ribosomal subunit. Tif1, Tif2, with sequence similarities to eIF-4A (Schmid and Linder, 1991), as well as SSL2 from *S. cerevisiae* (Gulyas and Donahue, 1992) were also found to be involved in translation initiation. Therefore, it is proposed that eIF-4A like RNA helicases are required for translation initiation in all eukaryotes.

A group of PRP proteins from *S. cerevisiae* were identified as RNA helicases based on their amino acid sequences. These proteins are involved in specific and distinct steps of pre-mRNA splicing. Among them, PRP2, PRP16, PRP22 and PRP43 belong to the DEAH-box protein family; while PRP5 and PRP28 are DEAD-box proteins. It was reported that PRP2 binds to a precatalytic spliceosome prior to the first step of splicing. It hydrolyzes ATP to cause a change in the spliceosome without the occurrence of splicing (Kim *et al.*, 1996). PRP28 has also been speculated to play a role in the first step of splicing by melting the duplex between U4 and U6 small nuclear RNAs (snRNAs), leading to the formation of

an active spliceosome (Chang *et al.*, 1997). PRP16 interacts transiently with the spliceosome during step two of the splicing reaction. It is thought that ATP hydrolysis promotes a conformational change and release of PRP16 from the complex, thus allowing the second cleavage-ligation step to take place. It has also been suggested that PRP16 may perform an additional role of regulating splicing fidelity by discarding aberrant lariat intermediates (Schwer *et al.*, 1991 and Burgess *et al.*, 1993). Schwer and Gross (1998) demonstrated that PRP22 also has two distinct functions in yeast pre-mRNA splicing. It can act on pre-assembled spliceosomes that are arrested after step one in an ATP-independent fashion or uses the energy of ATP hydrolysis to affect the release of mRNA from the spliceosome. Finally, PRP43 is believed to function late in the pre-mRNA splicing pathway to facilitate spliceosome disassembly (Arenas and Abelson, 1997).

A number of RNA helicases have been found to be involved in ribosome biogenesis including SrmB (Nishi *et al.*, 1988), DbpA (Boddeker *et al.*, 1997) from *Escherichia coli* and ten putative helicases from *S. cerevisiae*. SrmB overexpressed in *E. coli* was able to suppress a mutation in ribosomal protein L24 (Nishi *et al.*, 1988). L24 is essential in the assembly of the large ribosomal subunit; the mutant protein is defective in its interaction with 23S rRNA. Overproduction of SrmB may stabilize the L24 mutant protein by binding to a similar site on the 23S rRNA, or it may bind to an altogether different region and protect an unstable assembly precursor from degradation (Nishi *et al.*, 1988). DbpA was the first RNA helicase reported to have a specific RNA substrate, namely 23S rRNA, and was suggested to perform a general function in the assembly process of the 50S ribosomal subunit (Boddeker *et al.*, 1997; Fuller-Pace, 1993). In *S. cerevisiae*, Fal1p (Kressler *et al.*, 1997), Rok1p (Venema *et al.*, 1997), and Rrp3p (O'Day *et al.*, 1996) are required for formation of 18S rRNA . Dbp4p (Liang *et al.*, 1997), when overexpressed, rescues the 18S rRNA processing defect caused by U14 snoRNA mutants. Dbp7p (Daugeron and Linder, 1998), Dbp3p (Weaver *et al.*, 1997), Dbp6p (Kressler *et al.*, 1998), Drs1p (Ripmaster *et al.*, 1992), and Sbp4p (Sachs and Davis, 1990) are involved in the production of the 25S and

5.8S rRNAs, and Dob1p (dela Cruz *et al.*, 1998) is required for correct 3' end processing of the 5.8S rRNA.

Among the RNA helicases that have been identified in *E. coli*, RhlB, a member of the DEAD-box family, is a component of the degradosome which is involved in RNA turnover (Py *et al.*, 1996). It was demonstrated that the degradosome has an ATP-dependent activity that aids the 3'-5' degradation of structured RNA by the exoribonuclease polynucleotide phosphorylase (PNPase). Incubation of the degradosome with affinity purified antibody against RhlB inhibited the ATP-stimulated RNA degradation. These results suggest that RhlB acts by unwinding RNA structures that impede the processive activity of PNPase, and that RhlB is an important enzyme in mRNA turnover (Py *et al.*, 1996). Another *E. coli* DEAD-box protein CsdA was originally isolated as DeaD that suppresses a ribosomal protein S2 mutation (Toone *et al.*, 1991) and a cold-sensitive growth phenotype (Yamanaka *et al.*, 1994). This 70 kDa protein, was found to be specifically induced in *E. coli* when the culture temperature was shifted from 37°C to 15°C (Jones *et al.*, 1996). After the shift, CsdA is exclusively localized in the ribosomal fraction and becomes a major ribosomal-associated protein in cells grown at 15°C (Jones *et al.*, 1996). It was suggested that CsdA unwinds secondary structure in the 5' UTR of cold shock RNAs that are more stable thermodynamically under cold conditions and hence facilitates their translation (Jones *et al.*, 1996).

Several eukaryotic RNA helicases have been shown to play important roles in development and differentiation. For example, the nuclear protein p68 from human, an established RNA-dependent ATPase and RNA helicase, was demonstrated to be developmentally regulated and appears to correlate with organ differentiation/maturation in the fetus (Stevenson *et al.*, 1998). Vasa, another well characterized RNA helicase, was reported to be involved in localization of specific mRNAs to distinct sites within the *Drosophila* oocyte, which is an early and key step in establishing the anterior-posterior and dorsal-ventral axes (Tinker *et al.*, 1998).

Recently, a few RNA helicases have also been reported to be involved in oncogenesis. For example, human RNA helicase A was demonstrated to interact with the breast cancer specific tumor suppressor protein, BRCA1, and link it to the RNA polymerase II holoenzyme complex (Anderson *et al.*, 1998), while a DEAD box protein (DDX1) was found at elevated levels, with multiple copies, in a neuroblastoma and in some retinoblastoma cell lines (Godbout and Squire, 1996).

1.1.5 Substrate Specificity

RNA helicases have been found in all organisms and implicated in a wide range of cellular functions. Individual members of the family are highly conserved throughout evolution. This observation and the findings that relatively simple organisms, such as *S. cerevisiae* (Chang *et al.*, 1990) contain more than fifteen DEAD and DEAH proteins and that they do not complement one another suggest that each member has a highly conserved specific function that requires interaction with a specific RNA substrate. However, until recently there had been no reports of specific RNA sequence requirements by DEAD box proteins for *in vitro* ATPase and RNA helicase reactions except for DbpA from *E. coli* (Fuller-Pace *et al.*, 1993, Boddeker *et al.*, 1997). RNA helicases studied biochemically hydrolyze ATP in the presence of a variety of RNAs, including poly (A) and poly (U), and unwind a range of synthetic RNA substrates (Fuller-Pace 1994). *E. coli* DbpA, although able to unwind a variety of artificial dsRNAs, was shown to hydrolyze ATP only in the presence of bacterial 23S rRNA (Fuller-Pace, 1993). This was the first, and to date only identification of a specific RNA substrate for an RNA helicase. Nicol and Fuller-Pace (1995) investigated the nature of this specificity in depth and localized a region of 93 bases within the peptidyltransferase reaction center in the 23S rRNA that is both necessary and sufficient for complete activation of ATPase activity of DbpA. Furthermore, they have shown that the maintenance of secondary structure within this region was critical for activity. The authors hypothesized that DbpA may play roles either in establishing and/or maintaining the correct three-dimensional structure of the peptidyltransferase center in 23S

rRNA during ribosome assembly or in the peptidyltransferase reaction (Nicol and Fuller-Pace 1995).

However, in contrast, Boddeker *et al.* (1997) reported that aside from the 93 base region, four additional regions of the 23S rRNA can stimulate the ATPase activity of DbpA. These regions are spread across the 23S rRNA molecule and show no apparent consensus sequence. However, all of them are rich in stem-loop structures, and all of these 23S rRNA regions are either part of the functional center of the 50S ribosomal subunit or have been found to be related to ribosomal proteins playing leading roles in the assembly process (Boddeker *et al.*, 1997). The authors suggested that DbpA performs a general function in the assembly process of the 50S subunit rather than a specific role in peptidyltransferase activity.

Interestingly, it was also demonstrated that DbpA displays ATPase independent RNA helix destabilizing activity, suggesting that the ATPase and helicase activities are not necessarily coupled in all DEAD box proteins (Boddeker *et al.*, 1997). Moreover, the binding of RNA or ATP does not require the binding of the other. Therefore, they concluded that DbpA has two RNA binding domains, one that is 23S rRNA-specific and coupled to ATP hydrolysis while the second is non-specific for RNA (Boddeker *et al.*, 1997). So far, there is still no definite conclusion on RNA substrate specificity for DbpA, let alone all other RNA helicases.

1.1.6 RNA Helicases Function in Multi-subunit Complexes

It is known that the various cellular processes that RNA helicases are involved in are usually accomplished in protein and RNA complexes. Biochemical studies have shown that only a small number of the identified helicases are RNA-dependent ATPases and ATP-dependent RNA helicases. Moreover, none of them except DbpA displays substrate specificity in *in vitro* reactions, thus raising the possibility that accessory proteins are required for the unwinding activity or substrate specificity.

The prototype RNA helicase eIF-4A exists both in a free form and as part of the eIF-4F complex, which contains eIF-4A, the cap-binding protein eIF-4E, and eIF-4G, a protein required for the structural integrity of the complex (Rozen *et al.*, 1990). eIF-4A in the complex exhibits higher unwinding activity than free eIF-4A. In both forms, eIF-4A's ATPase and helicase activities are stimulated by an additional factor, eIF-4B (Abramson *et al.*, 1987). eIF-4B is an RNA binding protein possessing a ribosome dependent ATPase activity (Methot *et al.*, 1994). A model has been proposed in which eIF-4F binds to the 5' cap of eukaryotic mRNAs via the eIF-4E subunit, and then eIF-4B joins the complex. Stimulated by eIF-4B, eIF-4A unwinds the mRNA locally, using ATP hydrolysis as an energy source (Pause and Sonenberg, 1993). This provides ssRNA along which the 40S ribosomal subunit can "Kozak" scan all the RNA for the first AUG which is the initiation codon in eukaryotic mRNAs. As a result, more efficient translation initiation is achieved.

As described above (Section 1.1.2), RhlB, a DEAD-box RNA helicase in *E. coli*, functions as part of the degradosome, a complex which contains four major proteins: the endoribonuclease RNase E, PNPase, RhlB and the glycolytic enzyme enolase (Py *et al.*, 1996). The fact that recombinant RhlB binds RNA but does not exhibit ATPase activity suggests that the enzymatic activity of RhlB requires interaction with other components of the degradosome. An RNA helicase is required as an integral component of the degradosome as PNPase cannot degrade duplexed RNA. The unwinding of stem-loops by RhlB would therefore ensure the rapid degradation of highly structured RNA by PNPase (Py *et al.*, 1996).

The PRP proteins involved in yeast mRNA splicing provide other good examples of DEAD box proteins that interact with their RNA substrates as part of a large protein/RNA complex, in this case the spliceosome. Recently, the yeast DEAD-box protein, Dbp5p, was found to be associated with nuclear pore complexes assisting RNA export (Snay-Hodge *et al.*, 1998). The above evidence supports the idea that RNA helicases frequently function in multi-subunit complexes to play roles in various cellular processes.

1.1.7 Multimeric State of Helicases

An important aspect of RNA helicase structure, which has not been considered explicitly in most discussions, is the oligomeric state of the functional helicase. Several DNA helicases have been examined for self-assembly states and all of them were demonstrated to form oligomeric structures. For example, the *E. coli* proteins DnaB (Reha-Krantz and Hurwitz, 1978), Rho (Finger and Richardson, 1982) and RecBCD (Dykstra *et al.*, 1984) and the SV40 large T antigen (Mastrangelo *et al.*, 1989) can form hexamers; while the *E. coli* proteins helicase III (Yarranton *et al.*, 1979) and Rep (Chao and Lohman, 1991), as well as the HeLa helicase (Seo *et al.*, 1991) form dimers. The oligomeric state of these helicases appears to be limited to either dimer or hexamer. However, since the oligomeric states and self-assembly equilibria of most helicases have not been examined, this conclusion may not be general. Furthermore, for most of the above DNA helicases, the oligomerization state of the functionally active form of the helicase is unknown. There is good evidence that the active forms of at least three helicases are oligomeric. The *E. coli* Rep protein is induced to dimerize upon binding single stranded (ss) or duplex DNA and the ssDNA-dependent ATPase activity of the Rep protein is also stimulated significantly upon dimerization (Chao and Louman, 1991). In a second case, the SV40 large T antigen forms a double hexamer when bound to its origin of replication in the presence of ATP (Mastrangelo *et al.*, 1989). Finally, the three-subunit composition of the RecBCD enzyme and the fact that the individual subunits do not support helicase activity also suggest that its active form is oligomeric.

Although, to date, there has been no report of RNA helicase with multimeric structures, this possibility cannot be ruled out. Recently, Cho *et al.* (1998) determined the first crystal structure of an RNA helicase from the hepatitis C virus. They found that the structure consists of three domains including a NTPase domain, an RNA binding domain and a helical domain that contains no beta strand. These three domains form a Y-shaped molecule. The RNA binding domain is distinctively separated from the other two domains forming an interdomain cleft into which single stranded RNA can be modeled (Cho *et al.*,

1998). A channel was found between a pair of dimers that exhibit the most extensive crystal packing interactions (Cho *et al.*, 1998). A stretch of single stranded RNA can be modeled with electrostatic complementarity into the interdomain cleft and continuously through the channel (Cho *et al.*, 1998). The authors hypothesize that some form of this dimer is likely to be the functional form that unwinds double strand RNA processively by passing one strand of RNA through the channel and passing the other strand outside of the dimer.

On the basis of these observations, it is possible that other RNA helicases that are monomeric under some solution conditions may assemble to active oligomeric forms upon binding nucleotide cofactors (Lohman, 1992).

1.2 CYANOBACTERIA

1.2.1 General Characteristics of Cyanobacteria

Cyanobacteria constitute the largest, most diverse, and most widely distributed group of photosynthetic prokaryotes. The origins of cyanobacteria date back almost to the beginning of life on earth. Fossils of the progenitors of these organisms are found in strata over three billion years old (Fay, 1983). Since their recognition as a biological group early in the 19th century, they have been treated as a class or division of algae with the common name of blue-green algae. When it was determined that they were in fact prokaryotes, efforts were made to classify them under the Bacteriological Code (Rippka *et al.*, 1979). Taxonomically, cyanobacteria are linked by having two fundamental characteristics: they are Gram-negative prokaryotes and they perform oxygenic photosynthesis. Unlike higher plants, the enzyme responsible for photosynthetic carbon fixation, ribulose 1,5-bisphosphate carboxylase (Rubisco) is contained within proteinaceous membrane-bound vesicles (carboxysomes) in cyanobacteria. Moreover, cyanobacteria produce only chlorophyll *a* but not chlorophyll *b*, and contain a diverse and unique group of light harvesting proteins, the phycobiliproteins. The cyanobacterial light harvesting system is therefore more closely related to chloroplasts from red algae (Stanier and Cohen-Bazire, 1977). They are accordingly distinguishable from the other major group of photosynthetic

bacteria, the purple and green bacteria, both by the nature of their photosynthetic pigment system and by their capacity to perform oxygenic photosynthesis. Currently, cyanobacteria are classified into five provisional sub-groups (Rippka *et al.*, 1979). Section I comprises unicellular forms that reproduce by binary fission or budding. Section II also includes unicellular cyanobacteria, but only those that reproduce by multiple fission or by both multiple fission and binary fission. The filamentous cyanobacteria belong to the remaining Sections. Section III groups the strains that do not differentiate heterocysts; heterocystous filamentous strains comprise Section IV; Section V accommodates the heterocystous strains that in addition have true branching filaments (Rippka *et al.*, 1979).

Cyanobacteria are extremely diverse in that their habitat range from freshwater to marine environments, to high temperature regimes, and terrestrial areas including shorelines, salt marshes and mangroves (Whitton and Potts, 1982), as well as tropical and polar soils (Fay, 1983). As a result, many of them are capable of adapting to changes in their environment. For example, some species modify their light-harvesting apparatus in response to light quality, a process referred to as chromatic adaptation; some change their nutrient uptake systems or cellular morphology when nutrient deficiency is encountered; some exhibit cell differentiation, producing specialized cells such as heterocysts during nitrogen starvation and akinetes during the stationary phase of growth. The diversity of cyanobacteria also lies in their DNA base composition and genome size. Their G+C content can range from 35 to 71% (Herdman *et al.*, 1979a) and the genome size ranges from $1.6X10^9$ to $8.6X10^9$ daltons (Herdman *et al.*, 1979b).

As simple bacteria, cyanobacteria seem to be the obvious organisms of choice as a model system for the study of such fundamental processes as oxygen-evolving photosynthesis and nitrogen fixation. Numerous cyanobacterial species have been isolated as axenic cultures which grow in defined media (Rippka, 1988). The cells are small and exhibit rapid growth in liquid or on semi-solid media on which some species will grow as individual colonies (Rippka, 1988). In recent years great strides have been made in developing genetic systems for the analysis of various aspects of cyanobacterial physiology

and development. Transformation, electroporation and conjugation systems provide effective means for gene transfer in diverse cyanobacterial strains (Thiel, 1994). In 1995, the genome of *Synechocystis* sp. PCC 6803 was completely sequenced, which provided a very informative database for the study of cyanobacteria (Kaneko *et al.*, 1995; CyanoBase web site) . The complete genome sequence of *Synechocystis* together with gene transfer techniques and the ability to clone and inactivate genes in cyanobacteria have opened the door to advanced studies of photosynthesis, nitrogen fixation, heterocyst development and metabolism in these unique and important prokaryotic microorganisms.

1.2.2 *Anabaena* sp. strain PCC 7120

Anabaena sp. strain PCC 7120 belongs to the genus *Anabaena* which is one of several genera that comprise the heterocystous filamentous group of cyanobacteria; Section IV in the Rippka *et al.* (1979) classification scheme. It is an obligate photoautotroph and it is capable of heterocyst formation and aerobic nitrogen fixation. Physical mapping of the genome showed that the chromosome size of this organism is 6.4 megabases and its DNA is resistant to cleavage by numerous restriction endonucleases (Bancroft *et al.*, 1989). This strain possesses three large plasmids (Bancroft *et al.*, 1989), six insertion sequences (Cai and Wolk, 1990; Thiel, 1994) and a number of repetitive sequences known as short tandemly repeated elements (Mulligan and Haselkorn, 1989). As mentioned previously, gene transfer, mutagenesis and gene reporter systems have been well established in *Anabaena* sp. strain PCC 7120. Therefore, this strain has become a model system to study oxygenic photosynthesis, nitrogen fixation, and heterocyst formation.

1.3 COLD SHOCK RESPONSE

All living cells possess the ability to respond to changes in the environment, such as temperature, pressure, osmotic stress, and oxygen availability. Acclimation or adaptation at the cellular level in response to temperature flux occurs by alteration in metabolic rate, intracellular pH, ion concentration, membrane composition, and changes in gene

expression. An increase in temperature results in a specific heat shock response, which is shared by all organisms from bacteria to mammals. The heat shock response is characterized by the synthesis of a set of highly conserved heat chock proteins (Hsps) (Craig, 1985). Cellular responses to a decrease in temperature are not as well studied, and a conserved set of cold-inducible proteins among all organisms has not been identified. However, many species of bacteria have a small (approximately 7 kDa) highly homologous, cold-inducible protein, CspA (see below). In addition, there are common mechanisms for adaptation that are used by most organisms in response to cold temperature. These include alterations in membrane fluidity and changes in the protein translation machinery of the cell.

1.3.1 Membrane-Associated Changes

A universal response to low temperature is a change in the lipid composition of membranes. Membranes are normally in a liquid crystalline form and will undergo a transition to a gel phase state when the temperature drops. Many organisms have developed mechanisms to compensate for the transition from the liquid crystalline to gel phase by changing the degree of saturation of the hydrocarbon chains of membrane phospholipids. Phospholipids with unsaturated fatty acids have lower melting points and a greater degree of flexibility than phospholipids containing saturated fatty acids. This type of response, known as homeoviscous adaptation, was first shown in *E. coli* (Sinensky, 1974).

There are a variety of mechanisms that can alter the membrane phospholipid composition in response to temperature change. After a shift to low temperature, aerobic bacteria convert preexisting saturated fatty acids to the unsaturated form. In *Bacilli* a decrease in temperature results in increased synthesis of membrane bound desaturase, along with increased stability of this enzyme (Fulco *et al.*, 1980). In *E. coli* cis-vaccenate is produced 30 seconds following a shift from 42 to 24°C (Garwin *et al.*, 1980a). The

increased synthesis of this fatty acid is attributed to increased activity rather than induced expression of the enzyme synthase II at low temperature (Garwin *et al.*, 1980a and b).

1.3.2 Cold Shock Response in *E. coli*

The most extensive research on prokaryotic low-temperature adaptation has been conducted with *E. coli*. In *E. coli*, a downshift in temperature from 37°C to above 4°C causes a transient inhibition of most protein synthesis, resulting in a growth lag called the acclimation phase (Thieringer *et al.*, 1998). During this acclimation phase, a total of 16 proteins (Csps) appear to be cold inducible in *E. coli* (Thieringer *et al.*, 1998), of which 12 have been identified. Among the cold shock proteins, there is a family of structurally related proteins, CspA, CspB, and CspG, which show the highest induction in response to cold shock. These proteins belong to the CspA protein family that consists of eight members (CspA to CspH) that are all small molecular weight proteins, which share between 29% and 83% identity with one another (Yamanaka *et al.*, 1998). Among them, only CspA, CspB, and CspG are cold inducible. CspA has been extensively investigated at the level of both its function and mechanism of induction at low temperature.

In addition to the CspA family, the other identified cold shock proteins include: RecA, which is involved in recombination and induction of the cold shock response (Walker, 1984); H-NS, a nucleoid-associated DNA-binding protein, which is required for optimal growth at low temperature (Dersch *et al.*, 1994 and Sugino *et al.*, 1977); GyrA, the α-subunit of the topoisomerase DNA gyrase (Sugino *et al.*, 1977); NusA, which is involved in both termination and antitermination of transcription (Friedman *et al.*, 1984); PNPase, which is an exoribonuclease (Donovan and Kusher, 1986); and Hsc68, a Hsp70 homologue (Lelivelt and Kawula, 1995). In addition, three cold shock proteins have been shown to be ribosome associated: initiation factor 2 (IF2) (Jones *et al.*, 1987); an RNA unwinding protein (CsdA) (Jones *et al.*, 1996); and ribosome-binding factor A (RbfA) (Jones and Inouye, 1996).

The cold shock proteins of *E. coli* can be loosely categorized into two groups. Class I proteins are expressed at an extremely low level at 37°C and are dramatically induced to very high levels after a shift to lower temperature. In contrast, class II cold shock proteins are present at a certain level at 37°C and are induced a few fold from their steady-state levels after a downshift in temperature (Thieringer *et al.*, 1998). Interestingly, the class I proteins CspA, CspB, CspG, and CsdA all have in common an unusually long 5' untranslated region (5' UTR). For example, the *cspA* mRNA contains a 159-base 5' UTR, which plays an important role in transcription attenuation and *cspA* mRNA stability through extensive secondary structures present in the 5' UTR (Thieringer *et al.*, 1998; Bae *et al.*, 1997).

The cold shock response is induced during the transient block in initiation of translation. Among the *E. coli* cold shock proteins, three ribosome-associated proteins are involved in translation: IF2 for the binding of the initiator tRNA to the 30S subunit (Gualerzi, and Pon, 1990); CsdA, a member of the DEAD-box family of helicases (Jones *et al.*, 1996); and RbfA, a 30S ribosome-associated protein (Jones and Inouye, 1996). Both CsdA and RbfA are required for optimal growth at low temperature, indicating that two de novo ribosome-associated proteins, as well as induced synthesis of IF2, are required for efficient ribosomal function at low temperature.

What could be the signal for the induction of a group of proteins upon cold shock, of which at least three are proteins involved in translation? It has been shown that the cold shock response is induced when ribosomal function is inhibited (Jones and Inouye, 1996). Blockage of ribosomal function either by cold-sensitive ribosomal mutations or by antibiotics, such as chloramphenicol (Vanbogelen *et al.*, 1990 and Jiang *et al.*, 1993), results in an induction of the cold shock response. The data indicate that the physiological signal for the induction of the cold shock response is inhibition of initiation of translation caused by the abrupt shift to lower temperature (Jones and Inouye, 1996). As stated in the cold shock ribosome adaptation model, cold shock proteins RbfA, IF2, and CsdA associate with

the subunits of the 70S ribosome to convert the cold-sensitive non-translating ribosome into a cold-resistant translating state (Jones and Inouye, 1996). The end result is an increase in cellular protein synthesis and growth at the end of the acclimation period.

How, then, is the blockage of ribosome function able to induce the cold shock response? In the case of CspA induction at low temperature, mRNA stabilization plays a major role (Goldenberg *et al.*, 1996, Brandi *et al*, 1996 and Fang *et al.*, 1997). It is not known at present how the mRNA is stabilized, however, because mRNA degradation mechanisms are tightly coupled with the ribosome (Py *et al.*, 1996), uncoupling of such mechanisms from the ribosome may be involved in the dramatic stabilization of mRNA as observed for the *cspA* mRNA. It is important to notice that the *cspA* mRNA is constitutively produced even at 37°C but cannot be translated as a result of its extreme instability. It may be possible that upon cold shock the ribosome function becomes impaired and the mRNA degradative machinery may be dissociated from the ribosome (Thieringer *et al.*, 1998). Furthermore, cold-induced changes in the secondary structure of the CspA mRNA may change its RNase susceptibility (Thieringer *et al.*, 1998).

The preferential synthesis of cold shock proteins indicates that cold shock mRNAs, unlike most other cellular RNAs, possess a mechanism to form the translation initiation complex at low temperature. mRNAs for *E. coli* class I cold shock proteins, including CspA, CspB, CspG, CsdA, and RbfA, are equipped with an extra ribosome-binding site called the downstream box (DB) in the coding region of the mRNA (Mitta *et al.*, 1997, See Figure 1.2). In the case of *cspA* mRNA the DB plays an essential role in translation at low temperature during the acclimation phase. By having the DB, the *cspA* mRNA may be able to bypass the requirement of new ribosomal factors for translation initiation at low temperature. In addition to the DB, the long 5'UTR of the *cspA* mRNA contains a unique sequence called the "cold box", the sequence of which is highly conserved in other *E. coli* cold shock genes (Jiang *et al.*, 1996). It is a presumed transcriptional pausing site and is involved in the autoregulation of *cspA* expression. Bae *et al.* (1997) hypothesize that

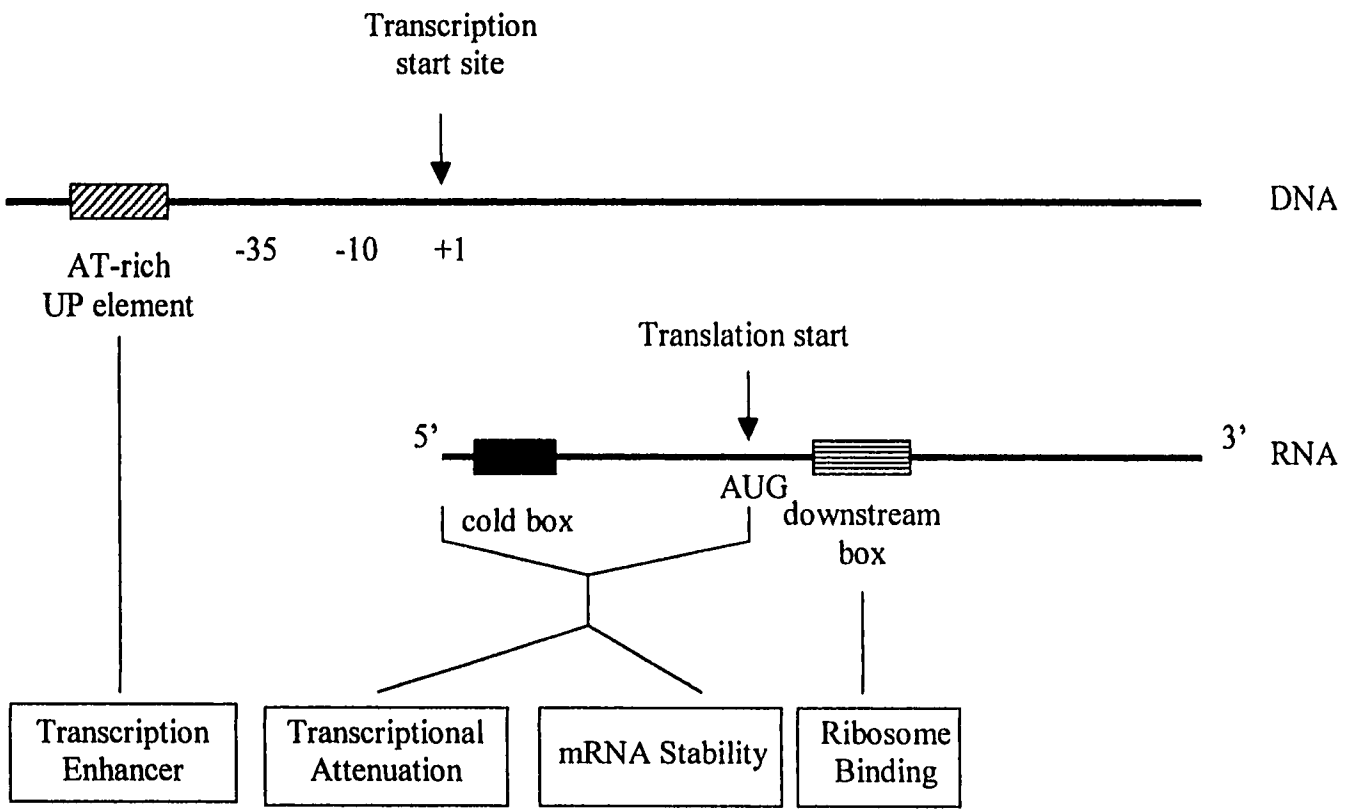


Figure 1.2 Regulatory elements implicated in the cold-induced regulation of CspA, the major cold shock protein of *E. coli* (Thieringer, *et al.*, 1998).

immediately following a temperature downshift, RNA polymerase somehow bypasses the pausing site in the *cspA* mRNA. However, as the cellular CspA concentration increases during the acclimation phase, CspA starts to bind its own mRNA to destabilize the elongation complex of RNA polymerase, resulting in attenuation of transcription. The transient production of CspA during the acclimation phase is indeed due to this autoregulatory mechanism.

The transcription of CspA at both 37 and 15°C is proposed to be controlled, in addition, by a transcriptional attenuation mechanism through CspC and CspE, two other non-cold shock members of the CspA family (Thieringer *et al.*, 1998). From deletion analysis of the *cspA* promoter region, the AT-rich sequence immediately upstream of the -35 region called the AT-rich upstream element (UP element) also plays an essential role in *cspA* transcription at both low and high temperatures (Mitta *et al.*, 1997). It is now apparent that the *cspA* expression is regulated at numerous levels, including transcription , mRNA stability, and translation (Figure 1.2).

In contrast to heat shock proteins in *E. coli* that require the synthesis of a specific heat shock sigma factor for their gene transcription, the cold shock induction of CspA does not require any new protein factors (Thieringer *et al.*, 1998). CspA can be produced upon cold shock by using previously existing resources in the cell at the time of temperature downshift. This seems to be the most secure way to induce a protein under any stress.

1.3.3 Cold Shock Response in Cyanobacteria

Cold-regulated growth of cyanobacteria has also been studied. So far, most of the work had focused on changes in the cell membrane. Cyanobacterial cells respond to a decrease in temperature by introducing unsaturated bonds into the fatty acids of membrane lipids. In this way, they compensate efficiently for the temperature-induced decrease in the molecular motion of the membrane lipids (Sato *et al.*, 1986; Murata and Wada, 1995). Fatty acid desaturases are the enzymes that introduce double bonds into the hydrocarbon chains

of fatty acids, and thus these enzymes play an important role during the process of cold acclimation of cyanobacteria (Wada and Murata, 1990). Four desaturases have been identified in *Synechocystis* sp. PCC 6803 including the DesA (Δ12 desaturase), DesB (ω3 desaturase), DesC (Δ9 desaturase) and DesD (Δ6 desaturase) (Dmitry *et al.*, 1997). The levels of the mRNAs of *desA, desB* and *desD* increased about 10-fold, but at different rates, upon a decrease in temperature from 34°C to 20°C whereas the level of *desC* transcript remained constant (Dmitry *et al.*, 1997). The increase in mRNA levels results from both the enhanced transcription and the increased stability of the mRNAs at low temperature. Sokamoto and Bryant (1997) have also identified three desaturases in *Synechococcus* sp. PCC 7002 that respond differently to temperature shifts. They showed that the steady-state mRNA abundance for the *desA* gene was three fold higher in cells grown at 22°C than in cells grown at 38°C (class II cold shock protein); *desB* transcripts were not detected at 38°C, but were abundant in cells grown at 22°C (class I cold shock protein); and *desC* mRNA levels were similar at both growth temperatures (not shock protein). The mRNA levels of each desaturase gene increased within 15 min of a temperature shift-down to 22°C, and mRNA levels recovered to basal levels within 15 min of a shift-up to 38°C. The half-lives of the *desA* and *desB* mRNAs were approximately four times longer in cells grown at 22°C than in cells grown at 38°C, but the *desC* mRNA had a similar half-life at both temperatures (Sakamoto and Bryant, 1997). These observations indicate that the expression of the genes for the desaturases is regulated by temperature in different ways.

Aside from the study of effects on cyanobacterial cell membrane composition at low temperatures, Sato has described several other cold shock induced genes. He isolated a low temperature-induced gene, *lti2* from *Anabaena variabilis* M3 that shows homology to α-amylases (1992). The level of *lti2* transcript increased 40-fold within an hour after a temperature shift from 38 to 22°C and then slowly decreased to a low steady-state level, which was similar to the level at 38°C. The ribosomal small subunit protein 21 and a family of cold-regulated RNA-binding proteins were also identified from the same strain (Sato,

1994). RNA binding proteins have been identified in various cyanobacteria, including *Synechococcus*, *Anabaena*, and *Chlorogloeopsis* (Mulligan and Belbin, 1995; Mulligan *et al.*, 1994; Sato, 1994). These proteins are small proteins with a single RNA recognition motif, containing both the highly conserved RNP1 (ribonucleoprotein 1) and RNP2 regions, and a short auxiliary motif (Mulligan and Belbin, 1995). Although the exact function of these proteins is not yet known, it was speculated that they may be involved in RNA processing and metabolism. Cyanobacteria do not have homologues of CspA family proteins, but the finding that most of these RNA-binding proteins are regulated by cold (Sato, 1995) suggests that they may play a role similar to the bacterial cold shock proteins as RNA chaperones to block the formation of secondary structures in RNAs at low temperature.

In our lab, an RNA helicase gene *crhC*, designated cyanobacterial RNA helicase Cold was insolated from *Anabaena* sp. strain PCC 7120 (Magee, 1997). Sequence analyses demonstrate that CrhC is most similar to RhlE (74.3% similarity, Magee, 1997), an *E. coli* RNA helicase whose function is unknown. Transcript analyses show that the expression of *crhC* gene was not detectable at 30°C but induced almost instantaneously at 20°C (Dr. Chamot, unpublished data), and the transcript remained at the same level as long as the cells continue to be grown at 20°C. In addition, the half-life of the transcript is significantly longer at 20°C than at 30°C (Dr. Chamot, unpublished data). The above evidence indicates that CrhC is a class I cold shock protein.

1.4 THESIS OBJECTIVES

In this thesis, I report the characterization of the RNA helicase protein, designated CrhC for Cyanobacterial RNA helicase Cold, from *Anabaena* sp. strain PCC 7120. Our attempts to investigate cyanobacterial RNA helicases were initiated for a number of reasons. Firstly, a cyanobacterial RNA helicase has not been extensively described in the literature. The characterization of such a protein would further support the idea that these

enzymes are wide spread in nature and required for essential cell processes. Secondly, the gene of the RNA helicase CrhC has been cloned and sequenced (Magee, 1997). Although sequence comparisons indicated that this protein is a putative RNA helicase, biochemical evidence is required to designate this protein as a true RNA helicase. Thirdly, Northern analysis performed in our lab demonstrated that the transcript of *crhC* gene is only present as a result of cold shock. It is therefore necessary to investigate the expression pattern of CrhC at the translational level. Finally, since a number of RNA helicases function as part of multi-subunit complexes, the localization of CrhC and the identification of the proteins interacting with CrhC will uncover the actual cellular function of this RNA helicase and lead to a greater insight of the cold shock response in cyanobacteria.

CHAPTER TWO
MATERIALS AND METHODS

2.1 GROWTH OF BACTERIA AND PHAGE

2.1.1 Maintenance and Growth of Bacteria

The bacteria used in this study, their relevant genotypes, and their sources are listed in Table 2.1. Throughout the text, bacteria harboring plasmids will be written with the strain first followed by the plasmid in parentheses [for example, JM109 (pGX29) denotes *Escherichia coli* strain JM109 containing plasmid pGX29]. *Anabaena* sp. strain PCC 7120 was maintained on BG-11 plates (Allen, 1968), containing 1% (w/v) Difco bacto agar in Coldstream incubators at 30°C under constant illumination (cool, white fluorescent light, 30 μmoles photons/m^2/sec). Liquid cultures of *Anabaena* sp. strain PCC 7120 in BG-11 were aerated by shaking at 250 rpm and bubbling with air. When cold shock treatment was required, liquid cultures were transferred to 20°C incubators for the indicated times.

E. coli DH5α and JM109 cells were maintained on LB media (Luria broth media containing 5 g/L yeast extract, 10 g/L bacto tryptone, 5 g/L NaCl). Solid media contained 1.2% (w/v) Difco agar. When harboring pGEX-2T or pGX29 plasmids (Table 2.2), JM109 cultures were grown in Trypticase soy broth (TSB) (Becton Dickinson) supplemented with 2% (w/v) glucose and maintained on plates containing 1.2% (w/v) Difco agar. Where required, ampicillin was added to 100 μg/mL.

2.1.2 Maintenance of Phage

M13 phage containing the T7 RNA polymerase gene used for induction of CrhC overexpression in *E. coli* was grown in *E. coli* JM109 cells in liquid LB media at 37°C, shaking at 100 rpm. M13 phage stocks were maintained at 4°C as clarified supernatants in LB media.

TABLE 2.1 Bacterial Strains

Strain	Relevant Genotype	Reference/Source	Use
Anabaena variabilis UTCC 387 (equivalent to *Anabaena* sp. strain PCC[a] 7120)	Wild type	University of Toronto Culture Collection (UTCC)	Study subject
Escherichia coli DH5α	*supE44 ΔlacU169 (ø80 lsvZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook *et al.*, 1989	Cloning
Escherichia coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F' [traD36 proAB$^+$ lacI^q lacZΔM15]*	Sambrook *et al.*, 1989	Protein overexpression

[a]Pasteur Culture Collection

TABLE 2.2: Parent Plasmids

Plasmid	Reference/Source	Selective Marker	Use
pBluescript KS+	Stratagene	Ampicillin	Cloning
pGX29	Magee, 1997	Ampicillin	Protein overexpression in *E. coli*
pRSETA	Invitrogen	Ampicillin	Protein overexpression in *E. coli*
pWM753	Magee, 1997	Ampicillin	Sequencing of 5'UTR of *crhC*
pGEM OM 1/2	Sonenberg, 1993	Ampicillin	RNA substrate for enzyme assays
pGEM 3	Sonenberg, 1993	Ampicillin	RNA substrate for enzyme assays

2.2 PURIFICATION OF DNA

2.2.1 Small Scale Plasmid Purification

Small scale plasmid DNA purification was performed by the TENS method (Zhou *et al.*, 1990), with minor modifications. An overnight culture (1.5 mL) was harvested in an eppendorf tube by microcentrifugation at 14000 rpm for 10 sec. The supernatant was decanted, leaving 50-100 μL and the cell pellet resuspended by vortexing. TENS (300 μL containing 10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.00, 1N NaOH; 0.5% [w/v] SDS) was added followed by 150 μL of 3 M sodium acetate, pH 5.2, and the mixture vortexed for 2 sec after each addition. Cellular debris was pelleted by microcentrifugation for 5 min. The supernatant was transferred to a clean tube, and DNA was precipitated by adding 900 μL of ice cold 95% ethanol, followed by microcentrifugation at room temperature for 4 min. The DNA was washed twice with ice cold 70% (v/v) ethanol, allowed to air dry, and resuspended in 150 μL sterile distilled H_2O.

2.2.2 Large Scale Purification of *E.coli* Plasmid DNA

Plasmid DNA was purified from 500 mL *E. coli* cultures using the QIAGEN plasmid Maxi kit according to the manufacturer's protocol with one modification. The cellular debris obtained after lysis was pelleted by centrifugation at 6000 rpm for 1 h at 4°C in a Beckman JA-14 rotor. The DNA was resuspended in sterile distilled H_2O at a final concentration of 1 μg/μL (Section 2.3.1).

2.3 MANIPULATION OF DNA

2.3.1 Determination of DNA Concentration

DNA concentration was determined by measuring absorbance at 260 nm. 1 μL of DNA sample was diluted in 1 mL distilled water and the absorbance of the solution at 260 nm was obtained. The concentration was calculated on the basis that one absorbance unit represents 50 μg of DNA.

2.3.2 Digestion and Gel Electrophoresis of DNA

DNA was digested with restriction enzymes obtained from Boehringer Mannheim, New England Biolabs, or Promega, according to the manufacturers' instructions.

DNA fragments were separated by electrophoresis through agarose gels (0.7-1.2% [w/v]) using either 0.5 X TBE (45 mM Tris-borate; 1 mM EDTA) or 1 X TAE (40 mM Tris-acetate; 1 mM EDTA) as the buffering system. One-fifth volume of 5 X DNA loading buffer (30% [w/v] sucrose; 0.125% [w/v] bromophenol blue; 5 mM EDTA, pH 8.0) was added to each sample prior to loading; RNase (at final concentration of 20 μg/mL) was also included when loading small-scale DNA samples. Gels were electrophoresed at constant voltage for various lengths of time. DNA was visualized by observing fluorescence under ultraviolet illumination after soaking the gel in an ethidium bromide solution (10 μg/mL) for 15 min.

2.3.3 Purification of DNA from Agarose Gels

DNA fragments were purified from 1 X TAE gels using the Geneclean II method (Bio 101) according to the manufacturer's instructions, with some modifications (Smith *et al.*, 1995). The DNA band was excised from the agarose, sliced into roughly 2 mm cubes and transferred to eppendorf tubes so that each tube contained less than 0.4 g gel slice. Three volumes of 6 M NaI was added, and the tubes incubated at 55°C for 5 min, mixing every 1-2 min. After the addition of 5 μL Glassmilk, the tube was incubated at 55°C for 5 min, mixing every 1-2 min. The Glassmilk was pelleted for 5 sec in a microcentrifuge at 14000 rpm and the supernatant removed completely. The pellet was washed three times with 250 μL of New Wash (50% [v/v] ethanol; 50% [v/v] buffer [20 mM Tris-HCl, pH 7.2; 0.2 M NaCl, 2 mM EDTA]). For DNA fragments smaller than 1.5 Kb, the pellet was washed by resuspension in New Wash and microcentrifugation for 5 sec. For DNA fragments larger than 1.5 Kb, the pellet was washed by diffusion for 5 min followed by

microcentrifugation for 5 sec. In both cases, all traces of New Wash were removed after the third wash. DNA was eluted from the Glassmilk by resuspending the pellet in 10-20 μL of sterile distilled H_2O and incubating at 55°C for 5 min, mixing every 1-2 min. Glassmilk was pelleted for 30 sec in a microcentrifuge. The supernatant containing the eluted DNA was stored at -80°C.

2.3.4 DNA Sequencing

DNA sequencing of the 5' untranslated region of *crhC* contained in pWM753 (Table 2.2) and pRSET29 (Table 2.3) was performed by the dideoxy chain termination method using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical). Prior to sequencing, double stranded plasmid DNA was denatured. For each 20 μL reaction, 3-6 μg dsDNA in 18 μL sterile distilled H_2O were mixed with 2 μL denaturing buffer (2 N NaOH; 2 mM EDTA) and incubated at 50°C for 5 min. DNA was precipitated at -80°C for 5 min after the addition of 14 μL 5 M ammonium acetate and 100 μL 95% ethanol, collected by microcentrifugation for 20 min at 4°C, and washed twice in 70% (v/v) ethanol. The final pellet was allowed to air dry and resuspended in 7 μL sterile distilled H_2O. Denatured template DNA was annealed to oligonucleotide primers (0.1 μg per reaction) for 12 min at 37°C in a 10 μL reaction volume containing 2 μL Sequenase buffer, cooled to room temperature for at least 30 min then placed on ice. Sequencing primers for this study are listed in Table 2.4. Template DNA was labeled at room temperature for 5 min in a reaction containing 1 μL 0.1 M DTT, 2 μL diluted labeling mix (1:5 in distilled H_2O), 0.5 μL [^{35}S]-dATP (Amersham Life Science), and 2 μL diluted Sequenase polymerase (1:8 in Sequenase dilution buffer). Termination reactions were performed by adding 3.5 μL of labeled template DNA to 2.5 μL of each termination mixture (ddATP, ddCTP, ddGTP, ddTTP), prewarmed to 37°C. Reactions were incubated at 37°C for 5 min and stopped by the addition of 4 μL stop solution.

TABLE 2.3: Plasmid Construction

Plasmid	Parent Plasmid	Selective Markers	Insert	Cloning Strategy	Use
pRSET29	pRSETA	Ampicillin	1.4 kb	B*am*HI/*Eco*RI fragment containing the ORF of crhC from pGX29	Protein overexpression

TABLE 2.4: Oligonucleotide Primers

Name	Sequence (5'-3')	Origin of Sequence	Use
T7 Primer	GTAATACGACTCTATAGGG	pRSETA	Sequencing
GWO43	CGTCCTGATAAGACAGCAG	*crhC* bp98 to 116 (antisense strand)	Primer extension

Polyacrylamide gels (6% [w/v]) were cast in 20X40 cm Bio-Rad Sequi-Gen II nucleic acid sequencing cells. A plug consisting of 10 mL 6% acrylamide stock (460 g/L ultrapure urea; 1 X TBE [90 mM Tris-borate; 2 mM EDTA]; 150 mL/L 40% acrylamide:bisacrylamide [Bio-Rad 19:1]); 50 μL 25% (w/v) APS and 50 μL TEMED was poured first and allowed to polymerize. Each gel was prepared by combining 50 mL 6% acrylamide stock and 75 μL 25% (w/v) APS, the mixture was degassed by vacuum filtration before adding 75 μL TEMED. Gels were allowed to polymerize at least 1 h prior to loading. Aliquots (3.5 μL) of the sequencing reactions, heated at 75°C for 2 min, were loaded using sharkstooth combs and the gels run at a constant power of 45 W in 1 X TBE buffer (prewarmed to 77°C) for 4-6 h. Gels were fixed in 5% (v/v) methanol; 5% (v/v) acetic acid for 30 min, transferred to Whatman 3MM paper, and dried under vacuum at 80°C for 1 h. DNA bands were visualized by autoradiography at room temperature.

2.3.5 DNA Ligation

DNA fragments, purified from agarose gels as described above (Section 2.3.2), were ligated to restriction endonuclease-digested plasmid cloning vectors at 15°C for 16-24 h using 1 unit of T4 DNA ligase and the manufacturer's supplied buffer (Boehringer Mannheim).

2.3.6 Bacterial Transformation

Competent *E. coli* DH5α and JM109 for transformation were prepared by treatment with rubidium chloride/calcium chloride solutions (Sambrook *et al.*, 1989). An overnight culture, grown in LB broth, was diluted 1:100 into fresh LB broth and grown at 37°C on a rotary shaker to an OD_{600} of 0.6. The culture was cooled at 4°C and the cells harvested at 4000 rpm for 10 min in a Beckman JA-14 rotor at 4°C. The cell pellet was resuspended in one-half volume ice cold 10 mM RbCl; 10 mM MOPS, pH 7.0 and incubated on ice for 10 min. The cells were centrifuged, the supernatant decanted and the pellet resuspended in

one-half volume ice cold 10 mM RbCl; 0.1 M MOPS, pH 6.5; 50 mM $CaCl_2$. After incubation on ice for 20-30 min, the cells were pelleted, resuspended in one-tenth volume RbCl; MOPS; $CaCl_2$ solution and DMSO added to a final concentration of 7% (v/v). Cells were dispensed into 0.8 mL aliquots and stored at -80°C. For each transformation, 200 µL of competent cells, thawed on ice, were added to a prechilled eppendorf tube and 1 µg DNA added. The mixture was gently mixed, incubated on ice for 30 min, and heat shocked at 42°C for 2 min. LB broth (1 mL) was added and the cells incubated for 1 h at 37°C on a rotary shaker. Aliquots (50-150 µL) were plated onto LBA (LB media containing 100 µg/mL ampicillin) plates and plates incubated at 37°C overnight. For detection of β-galactosidase activity for blue/white color selection of pBluescript KS^+ (Stratagene) clones, 50 µL of a 5:1 X-gal:IPTG (2% [w/v] X-gal; 100 mM IPTG) mixture was spread on plates prior to plating cells.

2.4 ISOLATION AND MANIPULATION OF PROTEIN

2.4.1 Cyanobacterial Protein Isolation

Exponentially growing cells (50 mL) were harvested by centrifugation at 10000 rpm for 7 min at 20°C in a Beckman JA-14 rotor for cold shocked cells or in a Janetzki T5 centrifuge in a 35°C warm room for warm-grown cells. The pellet was washed in 1/10 volume extraction buffer (20 mM Tris-HCl pH 8.0; 10 mM NaCl; 1 mM EDTA), transferred to eppendorf tubes and microcentrifuged at 14000 rpm at the appropriate temperature for 3 min. The washed cell pellet in each tube was resuspended in 1 mL ice cold extraction buffer containing 5 mM DTT and 1 mM PMSF, and lysed by sonication 4 X 1 min on ice with 1 min on ice between each sonication. For preparation of cell lysates for immunoprecipitation or native gel analysis, cells were lysed by vortexing in the presence of glass beads for 10 X 1 min with 1 min on ice between each vortexing. If the sample was to be analyzed by SDS-PAGE gel, NaCl was added to a final concentration of 100 mM and the lysate was microcentrifuged at 14000 rpm for 15 min at 4°C. If the sample was to be

used for immunoprecipitation or native polyacrylamide gel analysis, the lysate was directly microcentrifuged at 14000 rpm for 15 min at 4°C.

2.4.2 Precipitation of Proteins

Protein samples were mixed with 0.1 volume 10 mg/mL sodium deoxycholate (Sigma) and 0.1 volume 100% trichloro-acetic acid (Sigma) and the mixture was incubated on ice for 30 min. Proteins were collected by microcentrifugation at 14000 rpm for 30 min at 4°C and washed with 100% ice cold acetone. After air drying, the pellets were resuspended in appropriate volumes of 0.1 M Na_2CO_3, 0.1 M DTT.

2.4.3 SDS-Polyacrylamide Gel Electrophoresis of Proteins

Protein samples were separated electrophoretically on 10% (w/v) SDS-polyacrylamide gels using a Bio-Rad Mini-PROTEAN II electrophoresis cell. Resolving gels were made by combining 1.25 mL 30% (w/v) acrylamide : 0.8% (w/v) bisacrylamide (Bio-Rad); 450 µL 3 M Tris/HCl, pH 8.8; 37.5 µL 10% (w/v) SDS; 1.8 mL distilled H_2O; 1.875 µL 1.5% (w/v) APS. The gel was poured allowing space for a stacking gel of 1 cm. Immediately after pouring, isopropanol was layered on top of the acrylamide solution and the gel allowed to polymerize for 20 to 30 min. The isopropanol was poured off, and the gel surface washed thoroughly with distilled H_2O. The excess H_2O was removed by gently blotting with Whatman 3 MM paper. A stacking gel was made by combining 0.8 mL 30% (w/v) acrylamide: 0.8% (w/v) bisacrylamide; 1.25 mL 0.5 M Tris, pH 6.8; 50 µL 10% (w/v) SDS; 2.675 mL distilled H2O; 4 µL TEMED and 250 µL 1.5% (w/v) APS. The comb was inserted, the stacking gel poured and allowed to polymerize for 30 min. The wells were rinsed with 1 X running buffer (25 mM Tris; 0.192 M glycine; 0.1% [w/v] SDS) prior to loading.

Samples were prepared by adding 0.5 volume of loading buffer (125 mM Tris pH 6.8; 4% [w/v] SDS; 20% [v/v] glycerol, 10% [v/v] β-mercaptoethanol and 0.02%

bromophenol blue) and boiling for 2 min. The gels were electrophoresed in 1 X running buffer at 160 V until the bromophenol blue reached the resolving gel, after which voltage was increased to 200 V. Kaleidoscope prestained molecular weight standards (Bio-Rad), or unstained low molecular weight standards (Bio-Rad), treated identically to protein samples, were run on each gel.

2.4.4 Native Polyacrylamide Gel Electrophoresis of Protein

Native polyacrylamide electrophoresis of protein was carried out essentially as described for SDS-PAGE electrophoresis with the following modifications. Resolving gels contained 1.175 mL 30% (w/v) acrylamide (Life Technologies) : 0.5% (w/v) bisacrylamide (Aldrich Chemical Company); 1.25 mL 1.5 M Tris pH 6.8; 0.6 mL glycerol; 2.125 mL distilled H_2O; 15 µL 10% (w/v) APS and 15 µL TEMED. Stacking gels were made by combining 0.835 mL 30% (w/v) acrylamide : 0.5% (w/v) bisacrylamide; 401.5 µL 1.5 M Tris pH 6.8; 0.6 mL glycerol; 3.18 mL distilled H_2O; 20 µL 10% (w/v) APS and 4 µL TEMED. One third volume of loading buffer (0.5 M Tris pH 6.8; 50% [v/v] glycerol, 0.08% [w/v] bromophenol blue, 0.1% [v/v] β-mercaptoethanol) was added to samples and the gels were subjected to electrophoresis in 1 X native gel running buffer (10 mM Tris, 77 mM glycine pH 8.3) at 4°C at 10 mA for 1 h followed by 20 mA until the loading dye migrated to the bottom of the gels.

2.4.5 Staining of Proteins in Polyacrylamide Gels

After electrophoresis, protein gels were stained with Coomassie Brilliant Blue in order to visualize polypeptides. Gels were fixed in a destaining solution of 30% (v/v) methanol, 10% (v/v) acetic acid for 10 min to remove SDS, and stained in a solution of 0.25% (w/v) Coomassie Brilliant Blue R250, 45% (v/v) methanol, 10% (v/v) acetic acid for 10 min. The staining solution was removed and the gel destained by incubation in destaining solution for 2-15 h.

2.4.6 Electroelution of Protein from Polyacrylamide Gels

Electroelution of proteins was performed using an Electrophoretic Elution System EE-04 (Tyler Research Instruments) according to the manufacturer's instruction. Dialysis membrane with a molecular weight cut off of 12000 was cut into 6-inch lengths, soaked in 1% (w/v) $NaHCO_3$ at 60°C for 1 h, rinsed with water, soaked in 0.1% (w/v) SDS at 60°C for 1 h, and washed with water. The membrane was cut into disks and stored in 0.1% (w/v) SDS, 0.1% (w/v) NaN_3 at room temperature. Protein containing gel slices were rinsed with water, sliced into 1 mm cubes, soaked in elution buffer (0.1% [w/v] SDS; 0.05 M NH_4HCO_3) for 5 min, and transferred to the large well of the electrophoretic elution cell fitted with dialysis membrane. The gel pieces were covered with soaking buffer (2% [w/v] SDS; 0.4 M NH_4HCO_3) containing 0.1% (w/v) DTT. Elution buffer was carefully overlayed on top of the soaking buffer and the air bubbles in the cross passage cleared. The elution cell was inserted into the elution tank and elution buffer added to just above the drain ports in each electrode chamber. Another 75 mL of elution buffer was added to the mixing chamber and the buffer was moved from the mixing chamber to the electrode chamber by a peristaltic pump ([Watson Marlon-503S] 3 mL/min per line). Air bubbles were carefully removed from under the elution cell caps. After the gel pieces had been soaked for 3-5 h, elution was performed at 50 v for 12-16 h with the cathode on the side of the gel loading well. Elution buffer was then replaced with dialysis buffer (0.02% [w/v] SDS; 0.01 M NH_4HCO_3) and elution-dialysis carried out at 80 v for 4-6 h. The elution cell was removed from the tank, all the buffer except that in the conical sample collection well was removed, and the remaining protein containing solution (150-200 μL) transferred to an eppendorf tube. The collection well was rinsed with 50 μL fresh dialysis buffer and the buffer combined with the above sample.

2.4.7 Generation of Polyclonal Antisera

Polyclonal antibodies against purified CrhC protein were generated in a rabbit injected with the antigen. Approximately 250 μg gel purified antigen was thoroughly mixed with an equal volume of Freund's Complete Adjuvant (Serva) in a total volume of 1.8 mL until a milky emulsion was obtained. Before injection, a small sample of pre-immune serum was obtained from the rabbit. The rabbit was injected at 4-week intervals for a total of 3 times. Incomplete Adjuvant was used for the last two injections. Ten days after the third injection, a small aliquot of serum was tested at various dilutions using Western blots containing 0.1 μg of purified antigen. Since a 1 : 2000 dilution of serum was sufficient to detect purified antigen, no further injection was necessary. Blood was collected, allowed to coagulate at room temperature for 1 h and centrifuged at 5000 rpm for 15 min. The supernatant was aliquoted and stored at -80°C.

2.4.8 Electroblotting of Proteins

Electroblotting of proteins was performed using an Electrophoretic Transfer System ET-10 (Tyler Research Instruments) according to the manufacturer's recommendations. All components including Whatman 3 MM paper and 0.45 micron nitrocellulose filters (Bio-Rad) were soaked in 1 X transfer buffer (25 mM Tris/HCl; 150 mM glycine pH 8.3; 20% (v/v) methanol) for 10 min. Polyacrylamide gels to be electroblotted were not stained after electrophoresis. A sandwich was assembled of Whatman 3 MM paper, gel, nitrocellulose, Whatman 3 MM paper such that the nitrocellulose faced the anode and the gel faced the cathode. Transfer was allowed to occur for 1 h at 52 mA at room temperature. The efficiency of transfer was determined by staining the gel after transfer.

2.4.9 Western Blot Analysis

Western analysis was carried out as described (Lane and Harlow, 1982). Nitrocellulose filters after electroblotting were incubated in 1 X Blotto solution (1 X TBS

[10 mM Tris/HCl, 500 mM NaCl, pH 7.5], 5% skim milk powder [DIFCO], 0.02% sodium azide) for at least 30 min. Primary antiserum against CrhC over expressed in *E. coli* was added at a 1:500 dilution. The blot was incubated overnight at room temperature with gentle shaking. The blot was washed in three consecutive steps for 5 min each in 1 X TBS, 1 X TBST (1X TBS, 0.05% Tween-20), and finally 1 X TBS. The blot was incubated in fresh 1 X TBS containing 1:1000 diluted secondary antiserum (Goat anti-rabbit IgG Horseradish Peroxidase Conjugate [CAPPEL]) at room temperature for 3 h and washed as above. Antibody complexes were detected by addition of a solution consisting of 25 mL 1 X TBS, 15 mg 4-chloro-1-napthol (Sigma) in 5 mL methanol, 15 μL 37% H_2O_2 (BDH) in the dark for 15-30 min. Developed blots were rinsed with dH_2O and wrapped in saran wrap.

2.4.10 Immunoprecipitation

Immunoprecipitation was performed as described (Sigma Catalogue) with modifications. Cyanobacterial cultures (50 mL) were harvested at log phase (OD_{750}=0.3-0.5) as described above (Section 2.4.1). The cell pellet was resuspended in liquid BG-11 medium in which the sulfate-containing constituents $MgSO_4$, $ZnSO_4$ and $CuSO_4$ had been replaced by an equal molarity of $MgCl_2$, $ZnCl_2$ and $Cu(NO_3)_2$ respectively. [^{35}S]-methionine (Amersham) was added to the medium to a final concentration of 1 μCi/mL. The culture was incubated at an appropriate temperature for 1.5 h, after which the cells were pelleted, resuspended in 1 mL lysis buffer (20 mM Tris pH 8.0; 10 mM NaCl; 1 mM EDTA, 1% Triton X-100, 1 mg/mL BSA, 1 mM PMSF) and lysed as described above (Section 2.4.1). Clarified lysate (200 μL) was diluted to 1 mL with dilution buffer (10 mM Tris; 150 mM NaCl pH 7.5; 1% Triton X-100, 1 mg/mL BSA), 5 μL polyclonal antiserum added, and the mixture incubated on ice for 1.5 h. Lyophilized protein A-Sepharose powder (0.0125 g) was swollen in 1 mL buffer A (20 mM NaH_2PO_4; 150 mM NaCl pH 8.0) without any mechanical stirring for 0.5-2 h at room temperature, washed twice with 20 volume of dilution buffer, and gently resuspended in 50 μL dilution buffer. The above 1:1

(v/v) slurry (50 μL) was added to the diluted lysate, mixed with gentle shaking for 1.5 h at 4°C, the Sepharose beads were pelleted by microcentrifugation at 14000 rpm for 5 sec, and the supernatant carefully removed. The beads were washed twice with 1 mL dilution buffer and once each with 1 mL TBS (10 mM Tris, 150 mM NaCl pH 7.5) and 1 mL 0.05 M Tris pH 6.8. Laemmli sample buffer (20-50 μL containing 2% [w/v] SDS; 10% [v/v] glycerol; 0.05 M Tris pH 6.8 containing 0.02% [w/v] bromophenol blue) was added to the sample gently, without splatter, and the sample heated at 100°C for 5 min. After 5 sec microcentrifugation at 14000 rpm, the supernatant was loaded onto an SDS-PAGE gel (Section 2.4.3). The gel was analyzed by autoradiography using a Kodak BioMax TranScreen LE intensifying screen for ^{35}S overnight at -80°C.

2.4.11 Far-Western Analysis

Far-Western Analysis was performed as described (Methot *et al.*, 1996). After electroblotting, immobilized proteins on nitrocellulose filters were denatured by incubating the membranes with 8 M urea in HBB buffer (25 mM HEPES/KOH, pH 7.5; 25 mM NaCl; 5 mM $MgCl_2$; 1 mM DTT) for 10 min. The proteins were renatured in situ by 1:2 progressive dilutions of urea in HBB buffer for 10 min each. The membranes were blocked with 5% skim milk in HBB buffer for 30 min and incubated overnight in hybridization buffer (20 mM HEPES/KOH, pH 7.5; 75 mM KCl; 2.5 mM $MgCl_2$; 0.1 mM EDTA; 1 mM DTT; 0.1% Nonidet P-40; 1% milk) containing 1 μg/mL purified ChrC. The membranes were washed in five consecutive steps for 10 min each in 1 X TBST, and developed as described previously (Section 2.4.9).

2.5 OVEREXPRESSION OF PROTEIN IN *E. coli*

2.5.1 CrhC Overexpression using pGEX-2T

2.5.1.1 Induction of CrhC Protein

A single colony of JM109 (pGX29) cells was inoculated into 5 mL TSB containing 2% glucose and 100 μg/mL ampicillin, and grown overnight at 37°C. The overnight culture was diluted 1:50 into 25 mL fresh media and grown at 37°C to an OD_{600} of 0.6. Protein expression was induced by addition of IPTG to a final concentration of 0.5 mM, and the cells grown for 3 h at 37°C for purification of inclusion bodies or at 20°C for purification of soluble protein. The cells were then harvested by centrigufation at 4°C at 8500 rpm for 10 min and the cell pellet stored at -20°C.

2.5.1.2 Batch Purification of CrhC Protein from Inclusion Bodies

The cell pellet from a 5 mL culture was thawed on ice, resuspended in 1 mL PBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 pH 7.3), and transferred to eppendorf tubes. Cells were lysed by sonication, 4 X 1 min on ice with 1 min on ice between each sonication, using a Braun-Sonic 2000 sonicator fitted with a needle probe at 40 watts. If cells were induced at 37°C, the fusion protein was present in insoluble inclusion bodies which were collected by microcentrifugation at 14000 rpm at 4°C for 15 min. The inclusion bodies were washed twice in 1 mL PBS containing 2% Triton X-100 (Sigma) and resuspended in 1 mL PBS containing 0.1% Sarkosyl (Sigma). The suspension was gently shaken at room temperature for 30 min, solubilizing the majority of the inclusion bodies, and the solution clarified by microcentrifugation at 14000 rpm for 5 min. Solubilized fusion proteins were cleaved by addition of an equal volume of thrombin solution (prepared by diluting thrombin [Sigma] to 0.05 cleavage unites/μL in PBS) and incubation at room temperature overnight with gentle shaking. After thrombin cleavage, protein samples were precipitated (Section 2.4.2), resuspended in 320 μL 0.1 M Na_2CO_3, 0.1 M DTT and 160 μL loading buffer (Section 2.4.3), and boiled for 2 min. The individual GST and CrhC polypeptides created by thrombin cleavage of the overexpressed fusion protein were separated from each other by SDS-PAGE gel electrophoresis. After the electrophoresis,

gels were stained in 0.25 M KCl, 2 mM DTT at 4°C for 5 min. The "white" colored band of CrhC protein was excised and subjected to electroelution.

2.5.1.3 Batch Purification of Soluble CrhC Protein

E.coli JM109 (pGX29) cells were lysed as described above (Section 2.5.1.2). If cells were induced at 20°C, approximately 70% of the fusion protein was present in the soluble portion of the total cell lysate. Soluble CrhC protein was purified from the GST fusion protein using thrombin cleavage directly on a Glutathione Sepharose 4B matrix (Pharmacia). 50% Glutathione Sepharose 4B slurry (200 μL) in PBST (PBS containing 1% Triton X-100) were added to cleared cell lysate and the fusion protein allowed to bind to the matrix by incubation with gentle shaking at room temperature for 1 h. Glutathione Sepharose 4B beads were collected by microcentrifugation at 4000 rpm for 5 min and washed three times by addition of 1 mL PBST. Approximately 4.5 thrombin cleavage units, diluted in 50 μL PBST, were added to the beads and incubated at room temperature with gentle shaking for 2 h. The beads were sedimented at 4000 rpm for 5 min and the eluate containing soluble CrhC protein retained.

2.5.2 CrhC Overexpression using pRSET System

2.5.2.1 Cloning *crhC* into pRSETA

pGX29 (Table 2.2) was digested with *Bam*HI/*Eco*RI, the 1.4 Kb fragment gel purified (Section 2.3.2), and ligated into *Bam*HI/*Eco*RI-digested pRSETA (Invitrogen, Table 2.2). The resulting plasmid, designated pRSET29 (Table 2.3), contains an in-frame translational fusion with the coding sequence for a six-Histidine tag present in the pRSETA plasmid. The translational fusion was confirmed by restriction enzyme analysis and partial DNA sequencing. *E. coli* JM109 was transformed with pRSET29, resulting in the strain JM109 (pRSET29).

2.5.2.2 Induction of CrhC Protein

A single colony of *E. coli* JM109 (pRSET29) was inoculated into 5 mL LB containing 100 μg/mL ampicillin, grown overnight, and diluted 1:50 into 25 mL fresh media. The diluted culture was grown at 37°C with gentle shaking for approximately 3 h to an OD_{600} of 0.6, induced with the addition of IPTG and M13/T7 phage which provides T7 polymerase for the overexpression to final concentrations of 0.5 mM and 1 X 10^9 pfu /mL, respectively. Cells were grown for a further 5 h at 20°C, harvested, and stored as a pellet at -20°C.

2.5.2.3 Batch Purification of soluble CrhC Protein

Frozen cell pellet, representing 5 mL culture, was thawed on ice, resuspended in 1 mL lysis buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 10 mM imidazole), and transferred to eppendorf tubes. Cells were lysed by sonication as described above (Section 2.5.1.2). Cleared lysate (4 mL) was added to 1 mL of 50% Ni-NTA slurry ([QIAGEN] prewashed with 2 X 1 mL lysis buffer) and mixed gently by shaking at 4°C for 1 h. The lysate-Ni-NTA mixture was loaded onto a column (Bio-Rad) and the flow-through collected. The column was washed twice with 4 mL wash buffer I (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 20 mM imidazole) and once with 1 mL wash buffer II (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 40 mM imidazole). The protein was eluted with 3 X 0.5 mL elution buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 250 mM imidazole), and stored in 50% (v/v) glycerol at -20°C.

2.6 BIOCHEMICAL ENZYME ASSAYS

2.6.1 ATPase Assays

ATPase assays were performed as described by Rodriguez and Carrasco (1993) with minor modifications. Approximately 0.2 μg of purified CrhC protein was added to a reaction buffer containing 20 mM HEPES/KOH pH 7.5, 5 mM magnesium acetate, 1 mM

DTT, 40 μM ATP and 15 μCi/mL [γ-^{32}P] ATP ([ICN] 4500 Ci/mmol) in a final volume of 20 μL. Where required, total RNA isolated from various organisms (gifts from other labs) was also added to the mixture. The reaction was carried out at 37°C for 30 min and stopped in ice by adding EDTA to a final concentration of 0.1 M. Aliquots (2 μL) were analyzed by polyethyleneimine-cellulose thin-layer chromatography (Fisher Scientific) with 0.15 M formic acid, 0.15 M LiCl (pH 3) as the mobile phase. Autoradiography at -80°C of the dried chromatographic plates served to locate the positions of ^{32}P-ATP and $^{32}P_i$. When kinetic analysis was performed, the concentration of Mg^{2+} varied from 0.2 mM to 7 mM and the amount of RNA substrate varied from 1 μg to 20 μg.

2.6.2 RNA Helicase Assays

2.6.2.1 Preparation of RNA Substrate

RNA substrate, RNA II, was generated as described by Pause *et al.* (1993) using pGEM3 (Promega) and pGEM-MO1/2 vectors (Table 2.2). As shown in a schematic diagram in Figure 2.1, RNA II contains a double-stranded region of 14 bp with 5'-terminal extensions of single-stranded tails of 27 nucleotides and 54 nucleotides. The nucleotide sequences of the two strands are as follows: 41-nucleotide strand, GAAUACAAGCUUGC AUGCCUGCAGGUCGACUCUAGAGGAUC; 68-nucleotide strand, GGGAGACCGGA AUUCCCCAUGGCUGACUAAUUUUUUUUAUUUAUGCAGAGGGGGGAUCCUC UAGAGUC (duplex regions are underlined). pGEM3 was linearized with *Bam*HI and transcribed with SP6 polymerase (Promega), yielding a 41-nucleotide transcript. pGEM-MO1/2 was linearized with *Hinc*II and transcribed with T7 polymerase (New England Biolabs), yielding a 68-nucleotide transcript. Transcriptions were performed using a Riboprobe *in vitro* Transcription System kit (Promega) according to the manufacturer's protocol. [α-^{32}P] UTP ([Amersham] 50 μCi; 3000 Ci/mmol) was used to produce the 41-nucleotide strand and the 68-nucleotide strand was made with unlabelled nucleotides. The

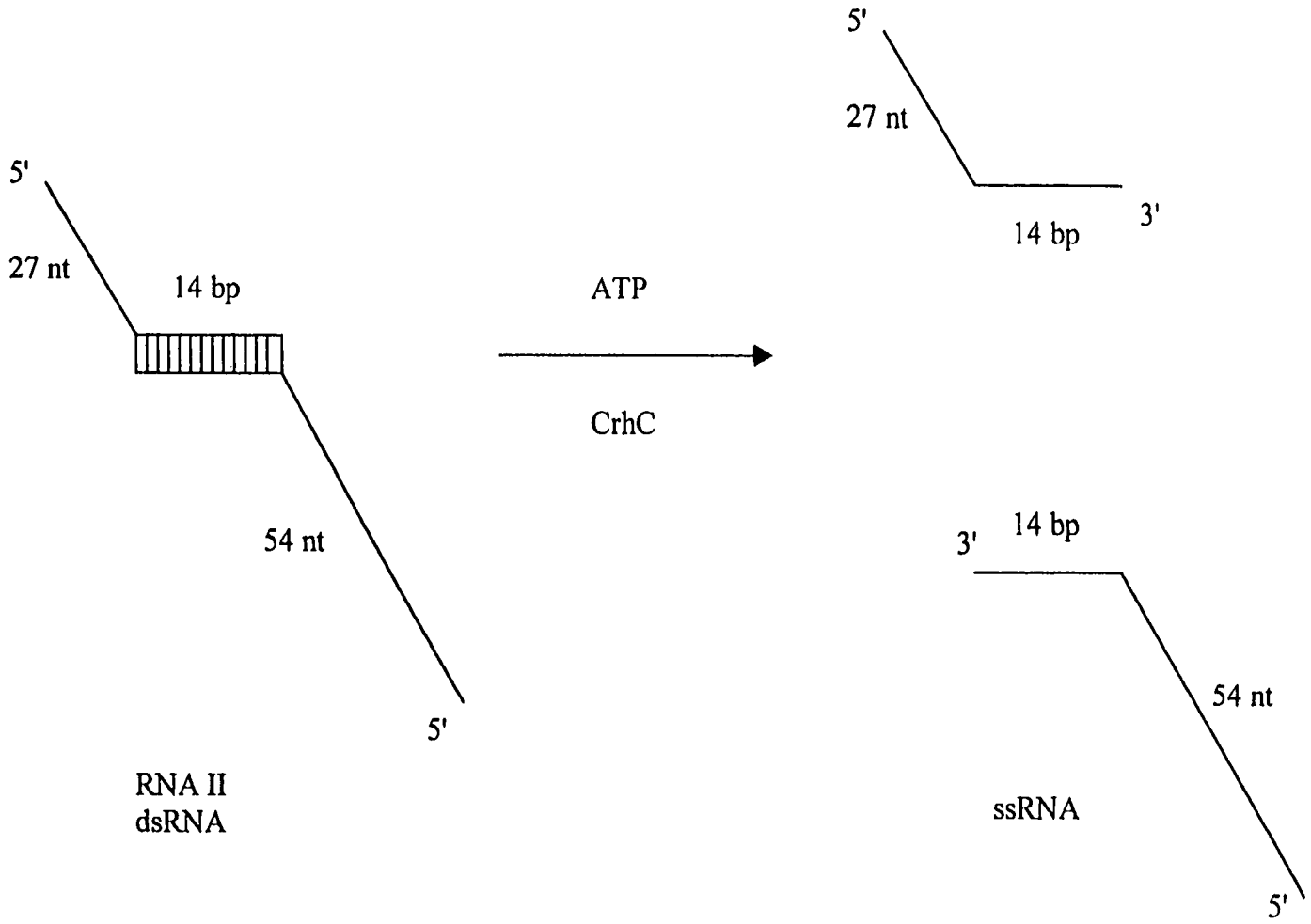


Figure 2.1 Schematic diagram of RNA II, and the process of unwinding by CrhC.

transcripts were separated on a denaturing 8 M urea-6% polyacrylamide gel, the bands were visualized by autoradiography or UV shadowing, excised, and eluted in 0.4 mL RNA elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS) overnight at 4°C. RNAs were phenol-chloroform extracted and ethanol precipitated. The two RNA transcripts were mixed in RNA annealing buffer (20 mM HEPES/KOH pH 7.2, 250 mM NaCl, 1 mMEDTA), heated at 95°C for 5 min, slowly cooled to 37°C, and incubated at 37°C for 2 h to allow hybridization. Duplex RNA was then purified on a native 8% polyacrylamide gel, excised, and eluted as described above.

2.6.2.2 RNA Helicase Assays

Standard RNA helicase assays were performed in 20 μL reaction mixtures containing 20 mM HEPES/KOH (pH 8.5), 3 mM $MgCl_2$, 1 mM DTT, 3 mM ATP, 20 units of RNasin (Promega), 200 μg/mL BSA, 2000 cpm dsRNA II substrate (Section 2.6.2.1), and 0.2 μg of purified CrhC protein. Reactions were stopped, after incubation at 37°C for 15 min, by the addition of 5 μL helicase stop solution (150 mM EDTA, 3% SDS, 0.25% Bromophenol blue, and 15% Ficoll). aliquots (10 μL) were loaded onto a 10% SDS-polyacrylamide gel (Section 2.4.3) and electrophoresed at 180 V for approximately 40 min. RNA was visualized by autoradiography and quantified on a Molecular Dynamics phosphorimager.

When kinetic analysis of the RNA helicase activity was carried out, the amount of CrhC added varied from 10 ng to 700 ng; the pH of the reaction buffer varied from 7.5 to 9.0; the reaction time varied from 5 min to 60 min, and the concentrations of $MgCl_2$ and ATP varied from 0.2 mM to 6 mM. Different types of nucleotides were also tested as energy source for the unwinding reactions at a final concentration of 3 mM.

2.6.3 RNA Binding Assays

RNA binding assays were performed essentially the same as the RNA helicase assays with minor modifications. SDS was removed from all the buffers so that the protein-RNA interaction was not disrupted. RNA-protein interaction was indicated by a mobility shift of the ^{32}P-labelled RNA II substrate, which was detected by autoradiography at -80°C.

2.7 PRIMER EXTENSION

An oligonucleotide primer beginning 98 nucleotides from the proposed translation initiation codon was designed (Table 2.4). The primer (50 pmol) was end labeled in a 10 μL reaction containing 1 μL 10 X phosphorylation buffer, 5 μL [γ-^{32}P] ATP ([ICN] 4500 Ci/mmol), 1 μL polynucleotide kinase ([Boehringer Mannheim] diluted 10 fold with RNase free distilled H_2O) at 37°C for 1 h. Unincorporated isotope was separated from the labeled primer by passage over a NucTrap probe purification column (Stratagene) and the amount of radioactivity incorporated determined by Cerenkov counting. End-labeled primer (6 X 10^5 cpm) was added to a 30 μL annealing reaction containing 10 μL 3 X aqueous hybridization buffer (3 M NaCl, 0.5 M HEPES pH 7.5, 1 mM EDTA pH 8), 30-50 μg total RNA extracted from *Anabaena* sp. strain PCC 7120 and RNase free distilled H_2O. The RNA and primer were annealed by heating the reaction to 80°C for 5 min, incubating at 65°C for 90 min, and slow cooling to room temperature. RNA was precipitated with 2 volumes of 95% ethanol on dry ice for 20 min, centrifuged at 13000 rpm for 5 min at 4°C, washed with 95% ethanol, and air dried. The precipitated RNA was resuspended thoroughly in 25.5 μL Reverse Transcriptase (RT) mix (prepared on ice by mixing 7 μL 2 mM dNTP, 5 μL 5 X RT buffer [Boehringner Mannheim], 0.5 μL RNase inhibitor [Boehringer Mannheim] and RNase free distilled H_2O). Primer extension was performed by the addition of 1 μL (20 units) AMV reverse transcriptase (Boehringer Mannheim) followed by incubation at 42°C for 1 h. The reaction was diluted to 300 μL by the addition of 1 μL glycogen (Boehringer Mannheim), 30 μL 3 M sodium acetate, and 243 μL 1 X TE

(10 mM Tris/HCl, pH 8; 1 mM EDTA, pH 8). The reaction products were precipitated and washed with 95% ethanol as described above, and the pellet resuspended in 5 μL loading buffer (DNA sequencing stop solution [Section 2.3.3]). pWM753 containing the entire open reading frame and its 5' UTR (Magee, 1997) was sequenced using the primer extension primer and loaded onto a 6% DNA sequencing gel (Section 2.2.4) together with 2.5 μL of the primer extension product. The dried gel was analyzed by autoradiography and the position of the transcription start site determined.

CHAPTER THREE
RESULTS

3.1 OVEREXPRESSION AND PURIFICATION OF CrhC

3.1.1 Overexpression of CrhC in *E. coli*

crhC was isolated and sequenced in our lab as a putative RNA helicase (Magee, 1997). Efforts were made to overexpress this gene in *E. coli* for two reasons. Firstly, transcript analysis done in our lab demonstrated that *crhC* is only expressed in cold shocked *Anabaena* sp. strain PCC 7120 (Dr. Chamot, unpublished data). To extend this result to the translational level, a large amount of purified CrhC protein was needed to generate polyclonal antibodies for Western analysis. Secondly, *crhC* was identified as a putative RNA helicase on the basis of amino acid sequence similarity to known members of this class of enzyme. To fully establish this protein as a true RNA helicase, assays for RNA helicase activity must be performed using purified CrhC protein.

Two overexpression systems (pGEX [Pharmacia] and pRSET [Invitrogen]) were employed for this process. As shown in Figure 3.1 Panel A, *crhC* was cloned into pGEX-2T creating a plasmid that contains an in-frame translational fusion of glutathione-S-transferase (GST), from pGEX-2T, and the *crhC* open reading frame (Magee, 1997). This fusion protein could be purified from total *E. coli* polypeptides by binding of the GST moiety to a Glutathione-Sepharose 4B column. In the pRSET system, the *crhC* open reading frame without the 5' untranslated region was cloned in-frame downstream from a DNA sequence that encodes a twenty-nine amino acid sequence containing 6 histidine residues, called a His-Tag (Figure 3.1 Panel B). Upon expression in *E. coli*, the resulting amino-terminal polyhistidine-tagged protein can be affinity purified as a result of the His-Tag binding to a Ni-NTA column.

Restriction sites required to clone the *crhC* open reading frame into the pGEX-2T vector were created using a modified site-directed mutagenesis protocol by Zoller and Smith (Magee, 1997), and the resulting clone pGEX29 was confirmed by restriction

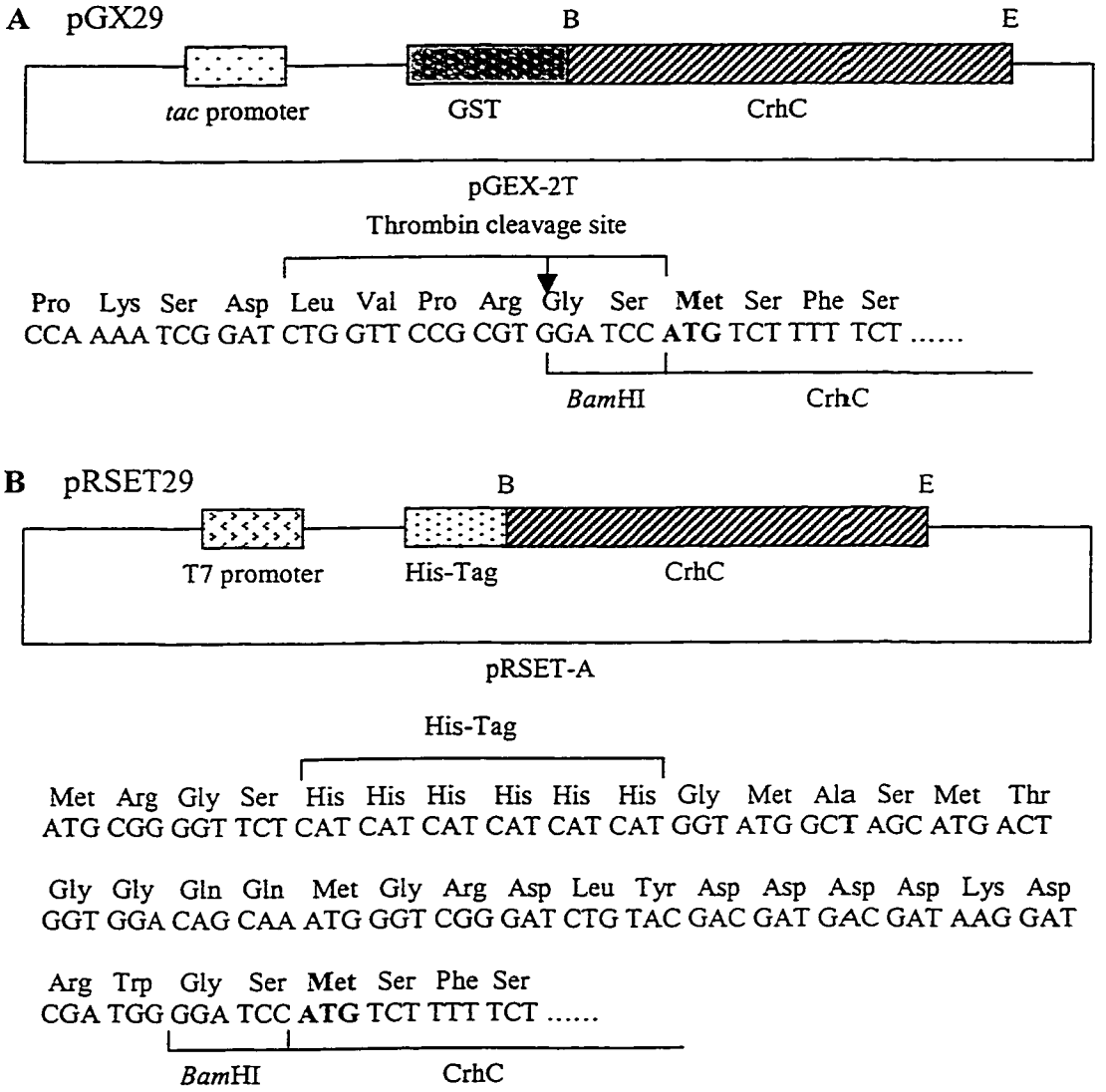


Figure 3.1. Construction of pGX29 and pRSET29. Panel A shows the construction of pGX29 and the sequence at the fusion region. The *crhC* gene as an *Bam*HI/*Eco*RI (indicated by "B" and "E" in the figure) fragment was cloned into pGEX-2T creating a plasmid that contains an in-frame translational fusion of glutathione-S-transferase (GST), from pGEX-2T, and the *crhC* open reading frame (Magee, 1997). Panel B shows the construction of pRSET29 and the sequence at the fusion region. The *crhC* gene as an *Bam*HI/*Eco*RI (indicated by "B" and "E" in the figure) fragment was cloned in-frame downstream from a DNA sequence that encodes a twenty-nine amino acid sequence containing 6 histidine residues (His-Tag).

enzyme analysis and partial DNA sequencing (Magee, 1997). Cloning the *crhC* gene into the pRSETA vector was easily accomplished, because the proper restriction sites had already been created during the cloning of pGEX29. The resulting translational fusion was also confirmed by DNA sequencing.

While all the cloning steps were performed in *E. coli* DH5α, pRSET29 was overexpressed in *E. coli* JM109 because the expression of *crhC* in pRSET29 was under the control of the T7 promoter and T7 RNA polymerase was provided by M13 phage that could not infect DH5α. When inducing *crhC*, both IPTG and M13 phage were added to log phase cells. For optimum expression, JM109 cells were not shaken rapidly before the addition of phage because vigorous shaking can shear the F pili and result in low infection rates.

3.1.2 Purification of CrhC from Inclusion Bodies

In both systems, when overexpressed in *E. coli* at 37°C, CrhC formed insoluble inclusion bodies that could only be solubilized in 0.1% Sarkosyl. In the pGEX system, the binding of the fusion protein to Glutathione-Sepharose 4B beads requires correct folding of GST. However, the presence of Sarkosyl apparently disrupted the secondary structure of GST and made it impossible to purify the GST/CrhC fusion protein by affinity chromatography. Since the production of polyclonal antibodies does not require protein in its native form, CrhC was purified from inclusion bodies. The GST/CrhC fusion protein was first solubilized in 0.1% Sarkosyl and the GST moiety removed by thrombin cleavage. CrhC was purified from the resulting protein mixture by electroelution of CrhC from SDS-PAGE gels (Figure 3.2). Purified CrhC protein was injected into a rabbit for a total of three times to generate polyclonal antibodies against CrhC as described previously (Section 2.4.7).

In the pRSET system, binding of the His-tag to the Ni-NTA column does not depend on protein conformation and therefore inclusion bodies solubilized in 0.1%

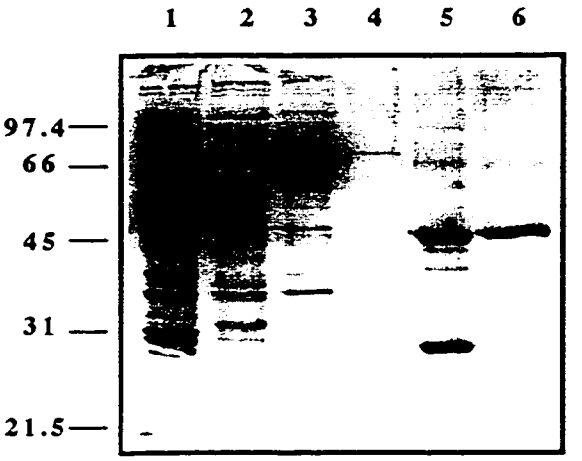


Figure 3.2. Overexpression and purification of CrhC from inclusion bodies using the pGEX system. Polyacrylamide gel (10%) showing various stages in the purification of CrhC protein: total cell lysate of uninduced *E. coli* JM109 (pGX29) (lane 1); clarified soluble portion of the total cell lysate (lane 2) and insoluble inclusion bodies (lane 3) of *E. coli* JM109 (pGX29) induced with 0.5 mM IPTG at 37°C for 3 h; 2% Triton X-100 wash of the inclusion bodies (lane 4); 0.1% Sarkosyl solubilized inclusion bodies cleaved with thrombin (lane 5); CrhC protein purified by electroelution (lane 6). Migration positions of molecular weight markers (Bio-Rad Low range) are indicated in kDa.

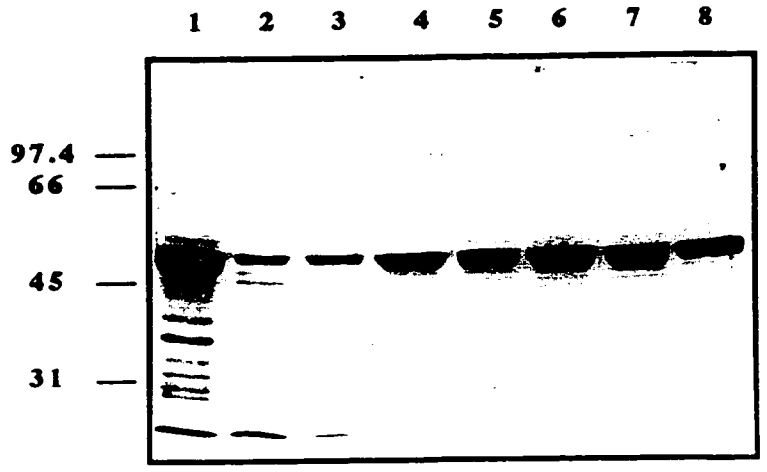


Figure 3.3. Overexpression and purification of CrhC from inclusion bodies using the pRSET system. Polyacrylamide gel (10%) showing various stages in the purification of CrhC protein: 0.1% Sarkosyl solubilized inclusion bodies of *E. coli* JM109 (pRSET29) induced with 1 mM IPTG in the presence of M13/T7 phage at 37°C for 3 h (lane 1); the flow-through collected after the incubation of solubilized inclusion bodies with Ni-NTA agarose beads at 4°C for 1 h (lane 2); the Ni-NTA agarose beads were washed with buffer containing 20 mM, 20 mM, and 40 mM imidazole (lanes 3, 4, 5); CrhC eluted from the Ni-NTA agarose beads with 3 X 1 column volume elution buffer containing 250 mM imidazole (lanes 6, 7, 8). Migration positions of molecular weight makers (Bio-Rad Low range) are indicated in kDa.

Sarkosyl could be purified by affinity chromatography. As shown in Figure 3.3, during the purification, although a fair amount of CrhC was eluted from the affinity column in the wash fraction, a significant amount of protein could still be obtained in the eluate fraction due to the high concentration of CrhC in inclusion bodies. However, CrhC purified in this fashion was not used for antibody production because by the time this method was completely established in our lab, the injections were already completed.

3.1.3 Purification of Soluble CrhC

In order to conduct biochemical enzyme assays, soluble and active CrhC protein was required. While solubilization of CrhC could be achieved in the presence of 0.1% Sarkosyl or 8 M urea, the recovery of active CrhC was extremely low. Therefore, it was necessary to optimize the conditions for soluble protein production. It has been reported that reducing culture temperature can suppress inclusion body formation and favor production of heterologous protein in a soluble form (Durairaj *et al.*, 1996). In this study, growth of cultures at 20°C after induction resulted in large amounts of CrhC present in the soluble portion of the total cell lysate using both the pGEX and pRSET systems (Figure 3.4 and Figure 3.5). It was estimated by SDS-PAGE that 70% of the CrhC protein was present in the soluble form at 20°C while more than 90% of the CrhC was present in inclusion bodies at 37°C. Growth at low temperatures may slow the rate of transcription from the T7 promoter, leading to production of CrhC in a soluble form.

Although soluble protein was generated with both systems, only a small amount of purified CrhC could be obtained with the pGEX system. As shown in Figure 3.4, the majority of the protein was eluted from the Glutathione-Sepharose 4B column in the flow-through fraction. It was likely that when expressed as the fusion protein GST/CrhC, the GST conformation was altered, which interfered with the binding of GST to the affinity matrix (Magee, 1997). As a result, the amount of protein obtained after the thrombin cleavage was not sufficient to perform enzyme assays.

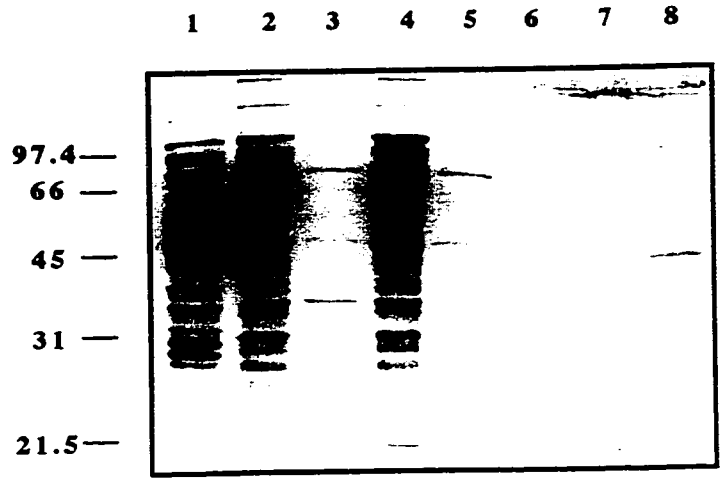


Figure 3.4. Overexpression and purification of soluble CrhC using the pGEX system. Polyacrylamide gel (10%) showing various stages in the purification of CrhC protein: total cell lysate of uninduced *E. coli* JM109 (pGX29) (lane 1); clarified soluble portion of the total cell lysate (lane 2) and insoluble inclusion bodies (lane 3) of *E. coli* JM109 (pGX29) induced with 0.5 mM IPTG at 20°C for 5 h; the flow-through collected after the incubation of the total cell lysate with Glutathione-Sepharose 4B beads at room temperature for 1 h (lane 4); the Glutathione-Sepharose 4B beads were washed three times with 1 X PBST (lanes 5, 6, 7); the CrhC/GST fusion protein was cleaved with thrombin and the purified CrhC was collected (lane 8). Molecular weight makers (Bio-Rad, Low range) are indicated in kDa.

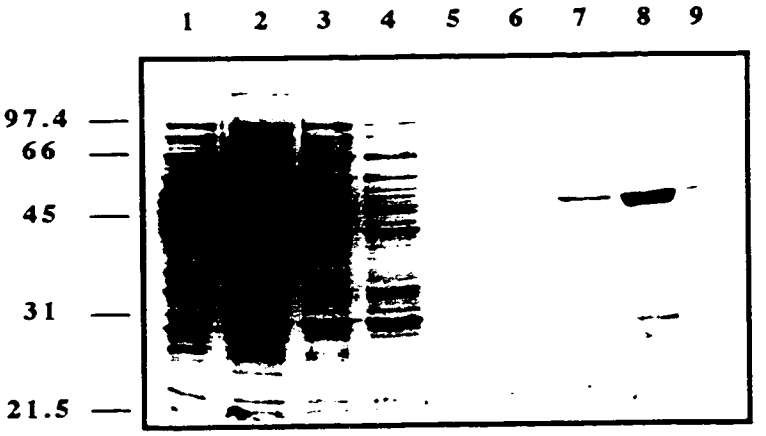


Figure 3.5. Overexpression and purification of soluble CrhC using the pRSET system. Polyacrylamide gel (10%) showing various stages in the purification of CrhC protein: total cell lysate of uninduced *E. coli* JM109 (pRSET29) (lane 1); clarified soluble portion of the total cell lysate of *E. coli* JM109 (pRSET29) induced with 1 mM IPTG in the presence of M13/T7 phage at 20°C for 5 h (lane 2); the flow-through collected after the incubation of the total lysate with Ni-NTA agarose beads at 4°C for 1 h (lane 3); the Ni-NTA agarose beads were washed with buffer containing 20 mM, 20 mM, and 40 mM imidazole (lanes 4, 5, 6); CrhC was eluted from the Ni-NTA agarose beads using 3 X 1 column volume elution buffer containing 250 mM imidazole (lanes 7, 8, 9). Migration positions of molecular weight makers (Bio-Rad, Low range) are indicated in kDa.

In contrast, when using the pRSET system, the interaction between the histidine tag and nickel column is not based on the secondary or tertiary structure; therefore, a sufficient amount of soluble and active protein could be generated by Ni-NTA column purification. As indicated in Figure 3.5, the majority of the CrhC could be specifically eluted from the affinity column. Efforts have been made to cleave the His-tag portion of the fusion protein with Enterokinase to generate pure CrhC. However, Enterokinase only functions at low salt concentrations, under which conditions, the CrhC protein aggregates and precipitates. Under the assumption that the His-tag does not exhibit RNA helicase activities, the affinity purified His-tag/CrhC fusion protein was used for all biochemical analysis.

During the purification and manipulation of CrhC protein, it was found that CrhC is conformationally unstable. Dialysis aimed at reducing salt concentrations or centrifugation methods designed to concentrate the protein resulted in aggregation and precipitation of CrhC. It was hypothesized that a certain ionic strength (> 50 mM) had to be present to maintain CrhC in its native form. CrhC stored in 50% (v/v) glycerol at -20°C remained active for at least one month.

3.2 IMMUNOLOGICAL ANALYSIS

3.2.1 Western Analysis of CrhC expression

It has been demonstrated in our lab that *crhC* is only expressed in cold shocked *Anabaena sp.* strain PCC 7120 (Dr. Chamot, unpublished data) at the transcriptional level. In order to study the expression of *crhC* at the translational level, Western analysis was performed using polyclonal antibodies against CrhC. Western blots of SDS-PAGE gels containing total cell lysates extracted from both warm-grown and cold shocked *Anabaena* sp. strain PCC 7120 showed that a 47 kDa protein was recognized by CrhC antibodies. In addition, it was present in the total lysate of cold shocked cells but not in that of warm-grown cells (Figure 3.6). This result confirmed that CrhC is only expressed in cold shocked

Anabaena sp. strain PCC 7120. On the majority of Western blots, a 120 kDa protein was also detected (Figure 3.6). The identity of this protein remains unknown.

3.2.2 Western Analysis of Native Protein Gels

Many helicases have been demonstrated to function in multi-subunit complexes. In an attempt to investigate the possibility that CrhC interacts with other proteins, native Western analysis was performed. Since the migration of proteins on native gels is not only affected by molecular weight, but also the structure and net charge of the protein, the association of a protein with other proteins affects its migration on native gels. Western blots of native protein gels indicated that purified overexpressed CrhC (obtained by thrombin cleavage of GST-CrhC protein produced in *E. coli*) had a significantly lower mobility than the CrhC protein from cold shocked *Anabaena* sp. strain PCC 7120 (Figure 3.7). CrhC is a positively charged protein with a theoretical pI of 10.8; therefore, pure CrhC migrated to a position near the negative electrode. Based on the result of native gel analysis we propose that CrhC is likely associated with other proteins that change the conformation of CrhC and/or mask positively charged groups present in CrhC.

3.2.3 Immunoprecipitation

To further investigate the possibility of CrhC interacting with other proteins, immunoprecipitation was performed. The rationale behind this method is that under native conditions, the antigen in total cell lysates will interact with its antibody and form an antigen-antibody complex, which can be purified. As a consequence, proteins associated with the antigen will be co-precipitated at the same time. Proteins immunoprecipitated from ^{35}S-methionine labelled cell lysates obtained from warm-grown and cold shocked cells, are shown in Figure 3.8. In cell lysate of cold shocked *Anabaena* sp. strain PCC 7120, several

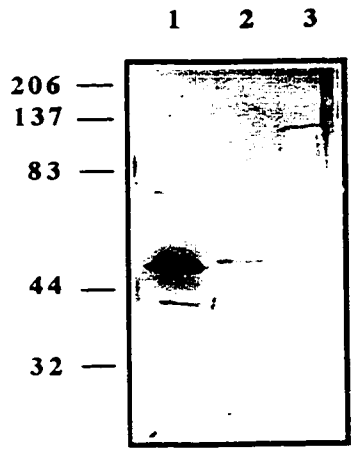


Figure 3.6. CrhC is only expressed in cold shocked cells. Log phase cultures of *A. variabilis* PCC 7120 grown at 30°C were either transferred to a 20°C incubator and incubated for 1 h (cold-shock), or shaken at 30°C for 1 h (warm). Total cell lysate containing 50 μg of proteins from cold-shocked cells (lane 2) and warm grown cells (lane 3) was subjected to Western analysis. A control of purified CrhC (obtained by thrombin cleavage of GST-CrhC protein produced in *E. coli* was included on the same gel (lane 1). Migration positions of molecular weight makers (Bio-Rad, Kaleidoscope) are indicated in kDa.

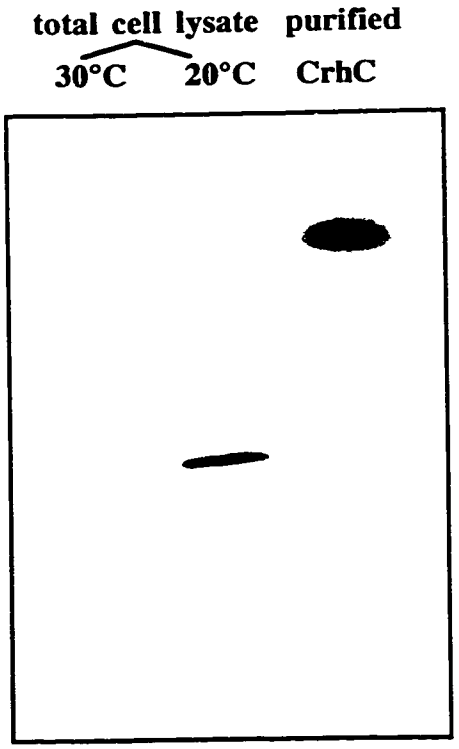


Figure 3.7. Migration of CrhC on native protein gels. Western blot of a native protein gel containing: total cell lysate of warm grown cells (30°C) and cold-shocked cells (20°C); Purified CrhC overexpressed in *E. coli* (obtained by thrombin cleavage of GST-CrhC protein) was included as a control.

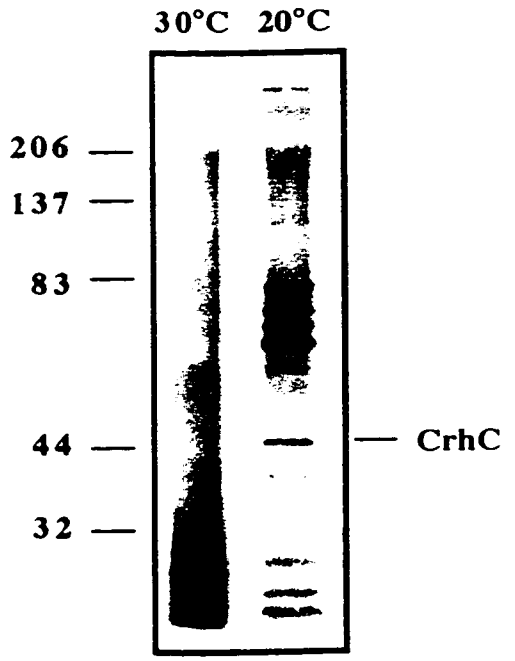


Figure 3.8. Immunoprecipitation of CrhC suggests that CrhC functions as part of a multi-subunit complex. Immunoprecipitations were performed on total cell lysates from warm-grown (30°C) and cold-shocked (20°C) *A. variabilis* PCC 7120 cells that were labelled with ^{35}S-Methionine. The antigen-antibody complexes were collected using protein A-Sepharose beads and analyzed by SDS-PAGE followed by autoradiography. Migration positions of molecular weight makers (Bio-Rad, Kaleidoscope) are indicated in kDa.

proteins co-precipitated with CrhC including four proteins within the size range from 60 kDa to 83 kDa, one protein of approximately 37 kDa, and three others in the size range from 20 kDa to 30 kDa. Cell lysate obtained from warm-grown cells, treated identically to cold shocked cells, do not contain proteins which are immunoprecipitated with CrhC antibodies. This is further evidence suggesting that CrhC functions in a multi-subunit complex.

3.2.4 Far-Western Analysis

Another technique to detect protein-protein interaction is Far-Western analysis. Unlike immunoprecipitation, which detects both direct and indirect interactions of proteins in a complex, Far-Western analysis only detects direct interactions between proteins. The theoretical basis of this method is that when incubating a Western blot containing total cell lysate, the purified protein of interest will interact with its target protein and remains on the blot; therefore, the target protein can be located by a regular Western analysis. The key to the success of this experiment is to ensure efficient binding of CrhC and its target protein. It was found that a denaturing-renaturing step between electroblotting and incubation of purified CrhC with the Western blot was a critical step required to produce clean, reproducible results.

A protein of approximately 37 kDa was consistently observed in Far-Western analysis. This protein was not, however, cold shock specific as it is present in both warm-grown and cold shocked cells (Figure 3.9). We are currently attempting to identify this protein.

3.3 BIOCHEMICAL CHARACTERIZATION OF CrhC

RNA helicases possess two major enzyme activities: RNA-dependent ATPase activity and ATP-dependent RNA unwinding activity. Although RNA helicase encoding genes have been described in many organisms, only a few RNA helicase proteins have been shown to catalyze both of the above reactions. To elucidate the biochemical activities of

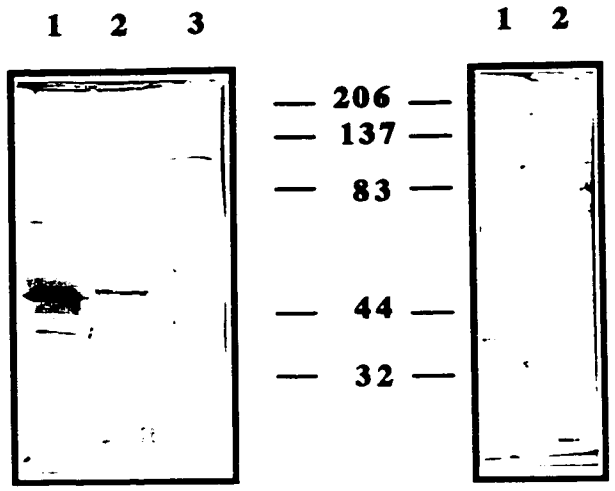


Figure 3.9. Far-Western analysis suggests that CrhC directly interacts with a 37 kDa protein. The blot on the left is a Western blot containing purified CrhC (lane 1), total cell lysate of cold-shocked (lane 2) and warm grown (lane 3) *A. variabilis* PCC 7120. The blot on the right is a Far-Western blot containing total cell lysate of cold-shocked (lane 1) and warm-grown (lane 2) *A. variabilis* PCC 7120 probed with purified CrhC protein. Both blots were developed together for the same length of time. Migration positions of molecular weight makers (Bio-Rad, Kaleidoscope) are indicated in kDa.

CrhC, a number of biochemical assays were performed including ATPase, RNA binding, and RNA helicase assays.

3.3.1 ATPase Assays

The theoretical basis of the ATPase assay involves thin layer chromatography detection of γ-^{32}P released from [γ-^{32}P] ATP in the presence of CrhC. In order to determine whether the ATPase activity of CrhC is RNA dependent, assays were performed in the presence and absence of RNA (Figure 3.10). CrhC only hydrolyzed a small amount of ATP in the absence of RNA. In contrast, the addition of total RNA (10 μg), extracted from both warm-grown and cold shocked *Anabaena* sp. strain PCC 7120, significantly increased ATPase activity, by approximately seven-fold over non-RNA containing reactions. These results demonstrated that CrhC is an RNA-dependent ATPase.

A series of ATPase assays were performed under various conditions to study the kinetics of the ATPase activity of CrhC. The effect of RNA was tested by adding increasing amounts of total RNA from *Anabaena* sp. strain PCC 7120 to the reactions. It was found that the optimum amount of RNA for stimulating the ATPase activity of CrhC was between 5 μg and 15 μg (Figure 3.11). 10 μg was chosen as the working concentration of RNA in all subsequent ATPase assays. The dependence of ATPase activity on the divalent cation, Mg^{2+}, was also determined where Mg^{2+} was added to reactions at concentrations ranging from 0.2 mM to 7 mM. The results showed that the optimum Mg^{2+} concentration for the ATPase activity of CrhC was 3 mM (Figure 3.12; Figure 3.13).

In an effort to obtain evidence that CrhC has a specific RNA substrate, total RNA isolated from different organisms was used in ATPase assays. It was shown that the ATPase activity of CrhC was stimulated by a variety of RNA at 10 μg (Figure 3.14 Panel A). The fact that RNA molecules without secondary structure, such as polyU, could induce the ATPase activity of CrhC seems to suggest that CrhC has no substrate specificity in

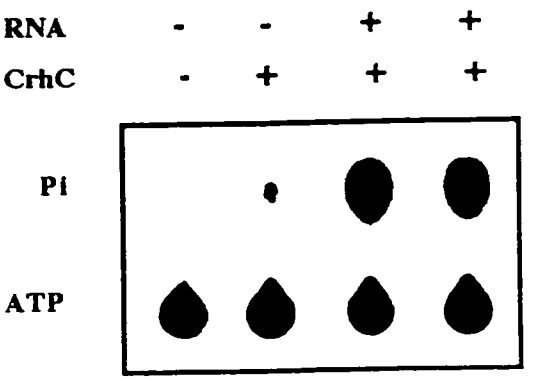
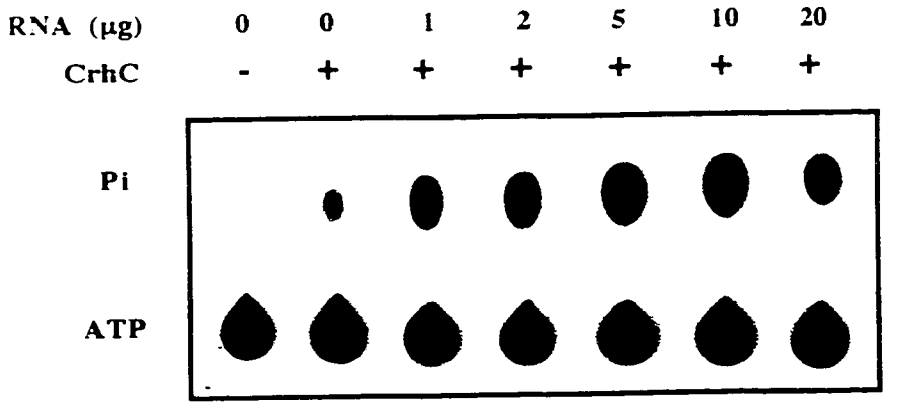


Figure 3.10. CrhC possesses RNA-dependent ATPase activity. ATPase reactions were performed with 0.2 μg purified CrhC under standard conditions as described previously (Section 2.6.1). An autoradiogram of the TLC plate is shown and the positions of ATP and released inorganic phosphate are indicated. The two lanes on the right are from reactions that contain 10 μg total RNA extracted from warm-grown and cold-shocked *A. variabilis* PCC 7120 respectively.

A



B

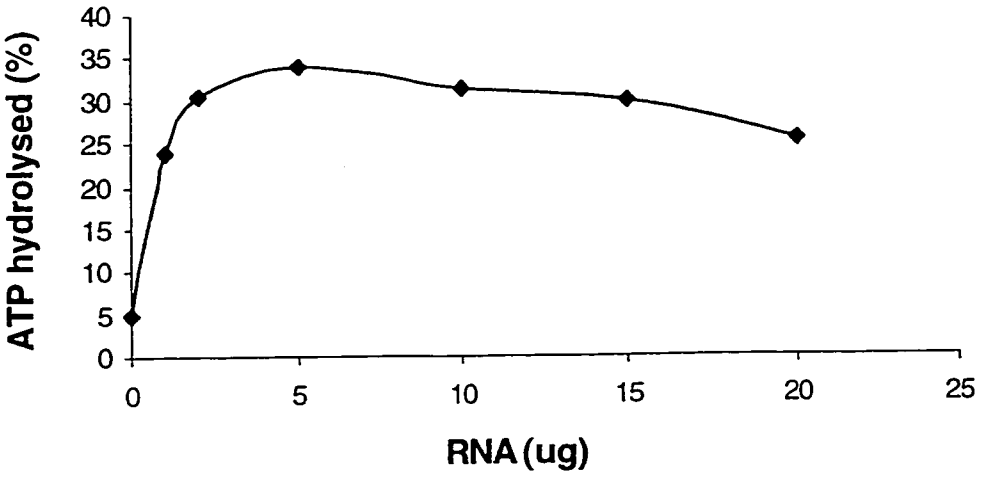


Figure 3.11. The effect of various amounts of RNA on the ATPase activity of CrhC. ATPase reactions were performed with 0.2 µg purified CrhC under standard conditions (Section 2.6.1). Various amounts total RNA isolated from cold-shocked *A. variabilis* PCC 7120 were added to the reactions as indicated above. Panel A shows the autoradiogram of the ATPase reaction, and its quantification is presented in Panel B.

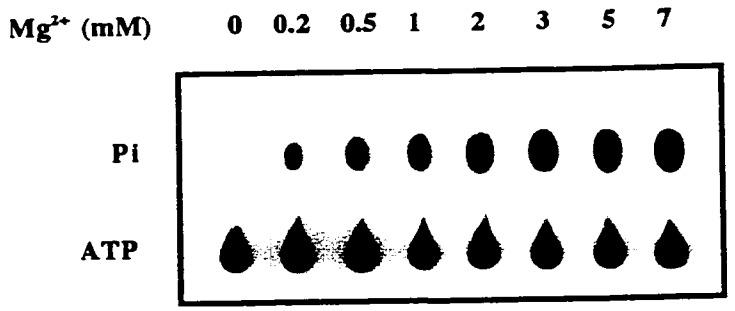
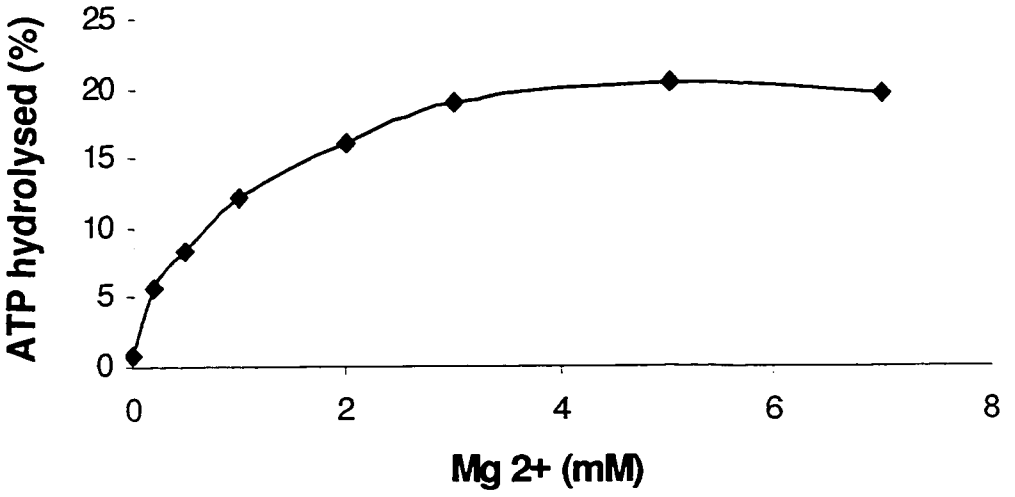


Figure 3.12. The ATPase activity of CrhC is Mg^{2+} dependent. ATPase reactions were performed with 0.2 μg purified CrhC under standard conditions in the presence of 10 μg total RNA extracted from *A. variabilis* PCC 7120. Mg^{2+} was added to various final concentrations as indicated above.

A



B

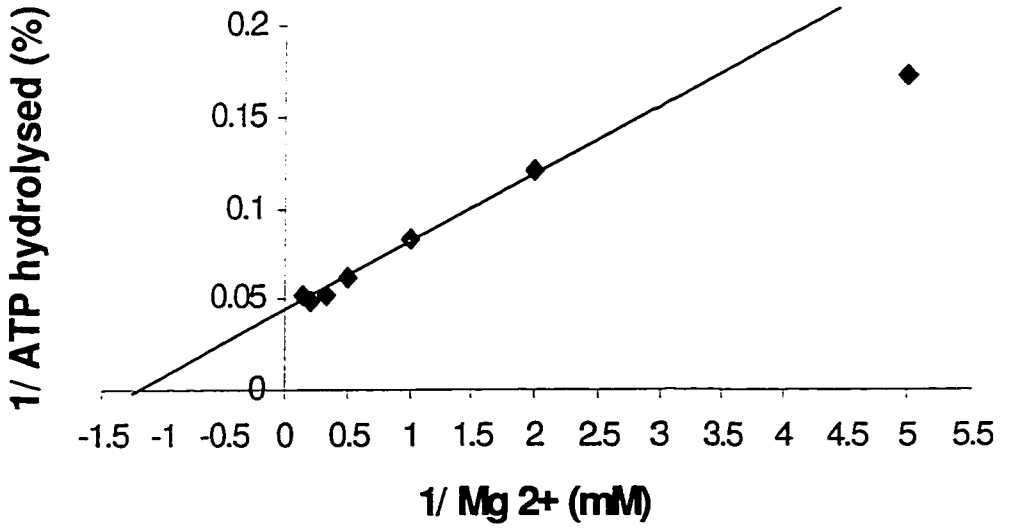
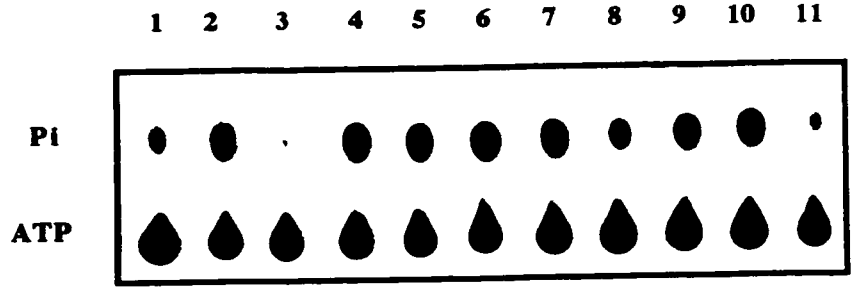


Figure 3.13. Kinetic analysis of the Mg^{2+}-dependence of the ATPase activity of CrhC. Panel A shows the quantification of the Mg^{2+}-dependent ATPase activity of CrhC. Panel B shows the Lineweaver-Burk plot of the ATPase activity versus the concentration of Mg^{2+} based on the results of Panel A. The x and y values represent the $1/[Mg^{2+}]$ μM and 1/the percentage of ATP being hydrolyzed, respectively.

A



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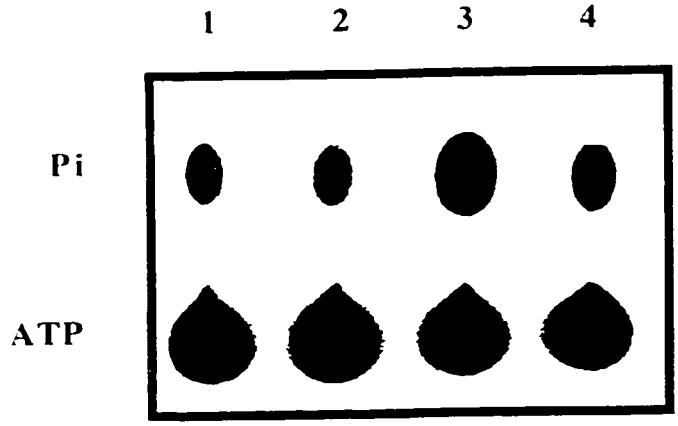


Figure 3.14. Substrate specificity of the ATPase activity of CrhC. ATPase reactions were performed with 0.2 μg purified CrhC under standard conditions in the presence of 10 μg RNA from various sources. Panel A: total RNA isolated from *S. cerevisiae* (lane 1), wheat (lane 2), *Streptomyces coelicolor* (lane 3), *Azotobacter vinelandii* (lane 4), *E. coli* (lane 5), cold-shocked and warm-grown *A. variabilis* sp. Strain PCC 7120 (lanes 9, and 10); poly U (lane 7) and tRNA (lane 8). As a control, a reaction containing no RNA (lane 11) was also included. Panel B: cold-shocked and warm-grown *A. variabilis* sp. Strain PCC 7120 (lanes 1, and 2); mixture of 16S and 23S rRNA (lane 3); mixture of poly A and poly U (lane 4).

in vitro assays. However, when partially degraded RNA molecules were added to ATPase reactions, no RNA stimulated ATPase activity was obtained, indicating that intact RNA molecules were required to induce the ATPase activity of CrhC and therefore, RNA secondary structure was essential for RNA-dependent ATPase activity. Moreover, it was also demonstrated that the ATPase activity of CrhC was preferentially stimulated by a mixture of purified 23S and 16S rRNA from *E. coli* when compared to total *A. variabilis* RNA (Figure 3.14 Panel B). This observation suggested that either or both rRNAs may specifically interact with CrhC, and this hypothesis should be tested with *Anabaena variabilis* 16S and 23S rRNAs.

3.3.2 RNA Binding Assays

RNA dependent ATPase activity suggests that RNA binds directly to CrhC thereby changing its enzymatic activities. RNA gel shift assays were utilized to determine the kinetics and specificity of RNA binding to CrhC. The target RNA for these studies was an artificially synthesized double stranded RNA II (dsRNA) radio-labelled with ^{32}P (Figure 2.1). An RNA gel shift experiment was done in which increasing amounts of purified CrhC were incubated with radioactively labeled dsRNA in the presence and absence of ATP (Figure 3.15). The results show that CrhC binds RNA in an ATP independent manner. In a 20 μL reaction, the amount of CrhC that could alter the migration of RNA molecules is as low as 20 ng, and this effect is not altered by the presence of ATP. Furthermore, the amount of RNA shifted was not in a linear relationship with the amount of CrhC added in the reactions, indicating a cooperative interaction.

3.3.3 RNA Helicase Assays

ATPase assays and RNA binding assays indicated that CrhC possessed RNA dependent ATPase activity and ATP-independent RNA binding activity. So, the energy released by ATP hydrolysis must be used for something else related to RNA instead of

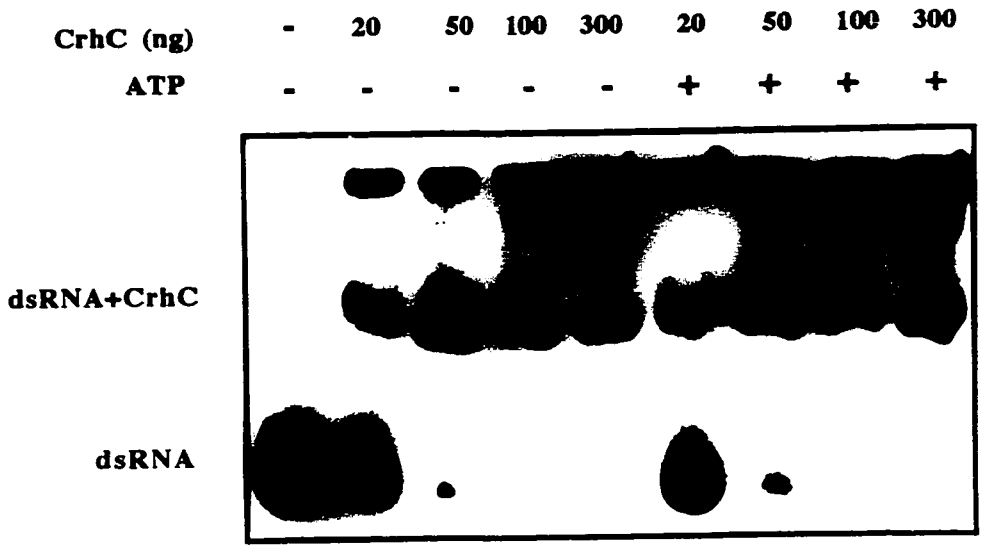


Figure 3.15. CrhC possesses ATP-independent RNA binding activity. RNA binding assays were performed using artificially synthesized dsRNA, RNA II. RNA II substrate (2000 cpm) was incubated with various amounts of purified CrhC in the presence and absence of 3 mM ATP (as indicated above), at 37°C for 30 min (Section 2.6.3). Reactions were analyzed on a 8% polyacrylamide gel eletrophoresis in 1 X TBE buffer. The migration positions of the dsRNA and the dsRNA/CrhC complexes are indicated.

RNA binding. This led us to carry out RNA helicase assays to see if the energy was required for RNA unwinding. Since ATPase assays indicated that CrhC did not have RNA specificity in *in vitro* biochemical enzyme assays, artificially synthesized dsRNA was used as the substrate for RNA helicase assays.

Interestingly, the RNA helicase activity of CrhC was found to be pH dependent. As shown in Figure 3.16, the highest activity was achieved at pH 9. It was suspected that the pH dependency of RNA unwinding was due to the high pI (10.8) of CrhC. pH 8.5 was used in all subsequent assays.

A series of standard assays were performed with the amount of CrhC ranging from 10 ng to 500 ng in a 20 μL reaction, in order to determine the effect of altering CrhC protein concentration on RNA helicase activity. It was found that the RNA helicase activity of CrhC was detectable at the 10 ng and was essentially completed at 50 ng (Figure 3.17). This result suggested that the RNA unwinding activity of CrhC is cooperative.

The time course of the unwinding activity of CrhC was determined. Assays containing 1 ng/μL CrhC indicated that the unwinding reaction proceeded in a linear fashion for 30 min and then entered a plateau phase (Figure 3.18).

The effects of ATP and Mg^{2+} on the unwinding activity of CrhC were also investigated. The unwinding activity showed a strict dependence on the presence of both ATP and Mg^{2+} (Figure 3.19 and Figure 3.21). The optimum concentration of ATP in RNA helicase reactions is 3 mM (Figure 3.20). The Lineweaver-Burk plot of the RNA unwinding activity versus the concentration of ATP indicated a Km for ATP of 71.4 μM (Figure 3.20). Quantification of the Mg^{2+}-dependency of the unwinding activity of CrhC demonstrated that approximately 80% of the dsRNA substrate was displaced at a Mg^{2+} concentration of 0.2 mM (Figure 3.22). The optimum concentration of Mg^{2+} was determined to be 1 mM. The Lineweaver-Burk plot (Figure 3.22) indicated a Km Mg^{2+} of 35.7 μM. The fact that the optimum concentration and Km Mg^{2+} is different from that of ATP suggested that Mg^{2+} was not required to complex ATP but to act independently as an enzyme cofactor.

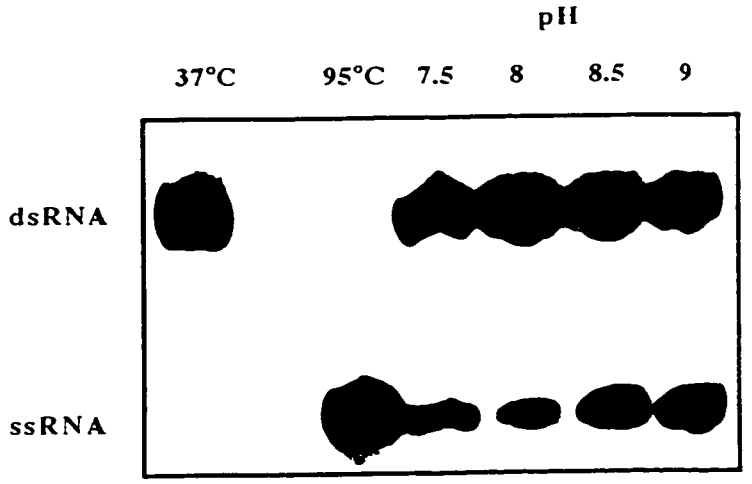
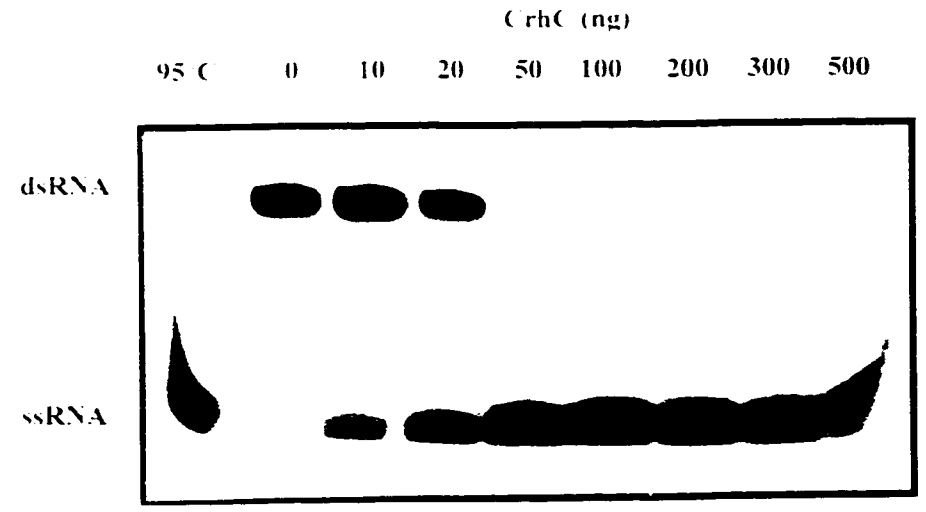


Figure 3.16. The RNA helicase activity of CrhC is pH dependent. An autoradiogram of the RNA helicase reactions. RNA helicase assays were performed under standard conditions as described previously (Section 2.6.2.2), except that the pH of the reactions varied from 7.5 to 9 as indicated above. Reactions were analyzed by SDS-PAGE followed by autoradiography. Controls including dsRNA, RNA II, incubated in the pH 9 reaction buffer in the absence of CrhC at 37°C, and the dsRNA heat-denatured at 95°C for 3 min were also loaded on the gel (the two lanes on the left), respectively.

A



B

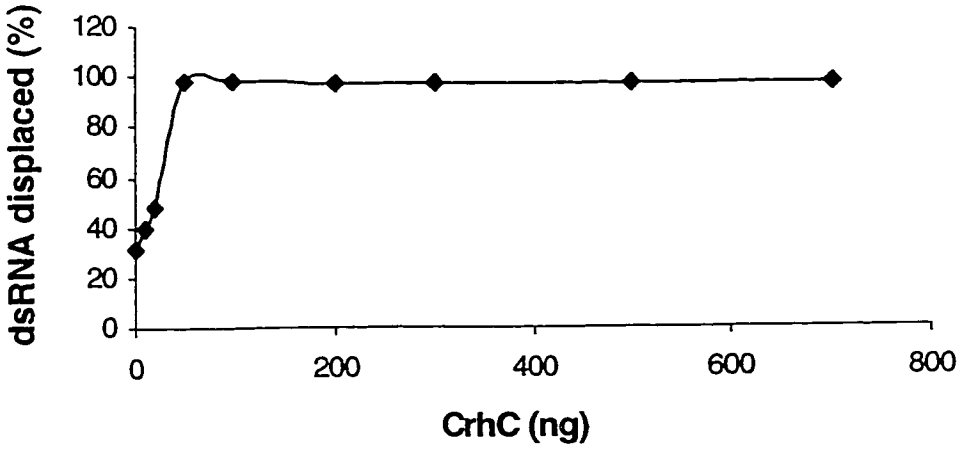
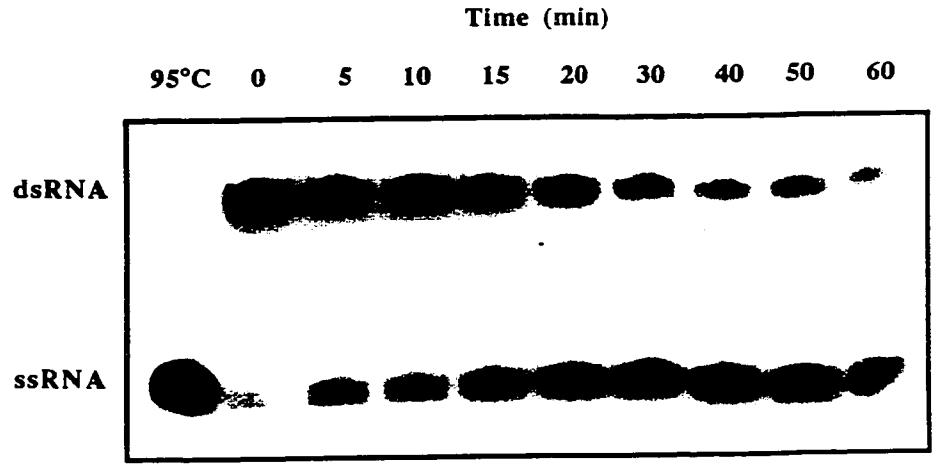


Figure 3.17. Influence of enzyme concentration on RNA helicase activity. The RNA helicase reactions were performed with 2000 cpm dsRNA substrate, RNA II, and the indicated amounts of CrhC under the standard assay conditions for 10 min (Section 2.6.2.2). A control of heat-denatured dsRNA was also included (the first lane on the left). Panel A shows an autoradiogram of the helicase reactions, and its quantification is presented in Panel B.

A



B

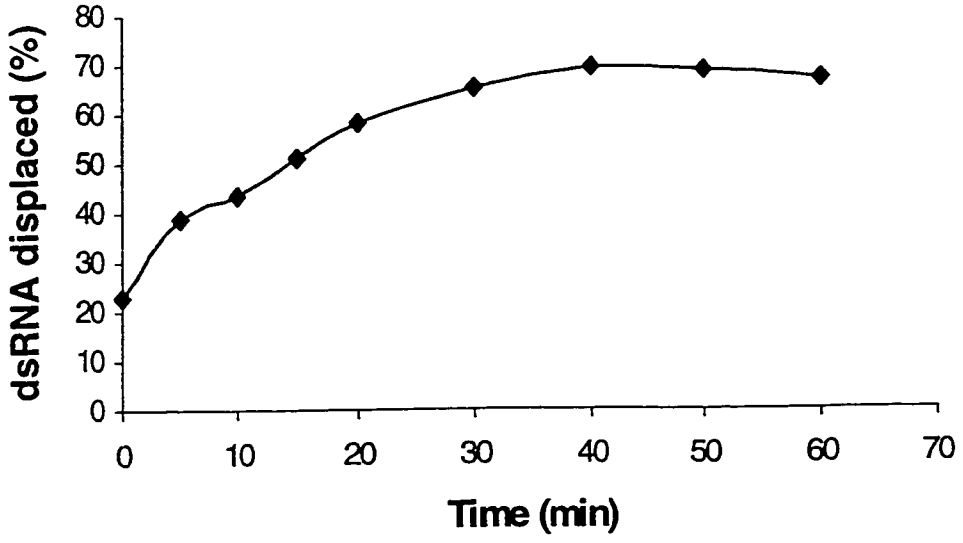


Figure 3.18. A time course of the RNA helicase reaction. RNA helicase reactions were performed with 20 ng purified CrhC and 2000 cpm dsRNA, RNA II, under standard conditions. Reactions were stopped after the indicated incubation period. Panel A shows the autoradiogram of the helicase reactions, which was quantified as shown in Panel B.

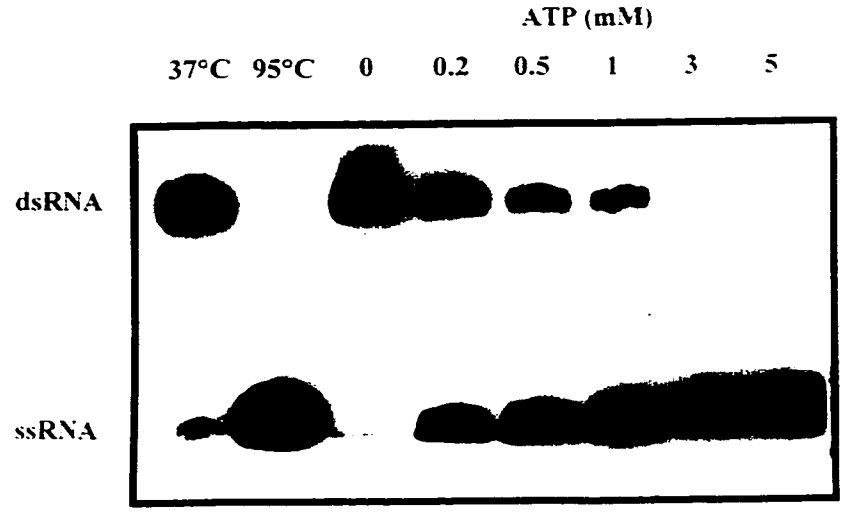
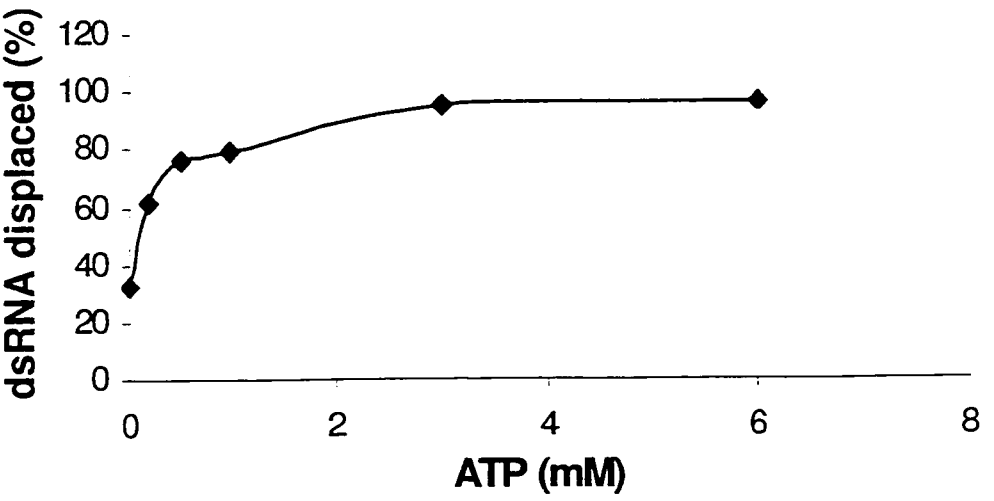


Figure 3.19. The RNA helicase activity of CrhC is ATP dependent. The helicase reactions were performed under standard conditions except that ATP concentrations varied from 0 mM to 5 mM as indicated above. Controls of native dsRNA and heat-denatured dsRNA were included (the left two lanes).

A



B

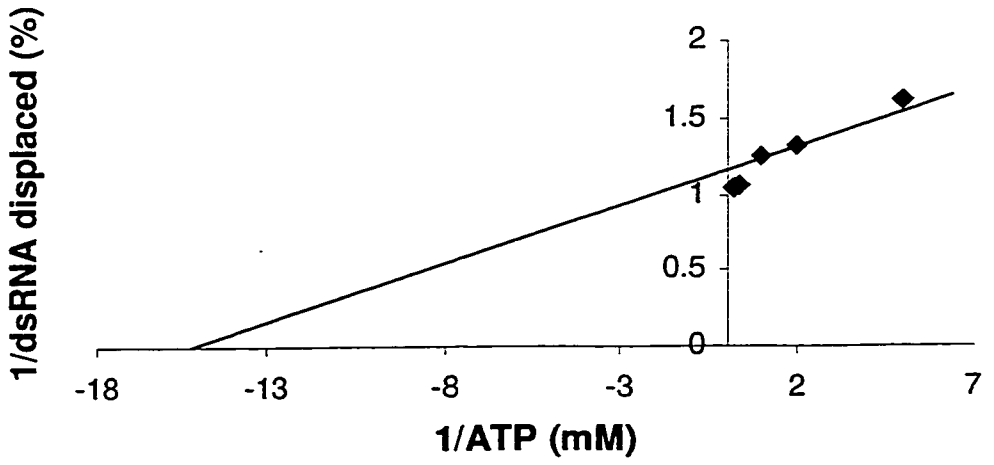


Figure 3.20. Kinetic analysis of the ATP-dependence of the RNA unwinding activity of CrhC. Panel A shows the quantification of the ATP-dependent RNA helicase activity of CrhC. Panel B shows the Lineweaver-Burk plot of the RNA helicase activity versus the concentration of ATP based on the results of Panel A. The x and y values represent the 1/[ATP] μM and 1/the percentage of dsRNA being displaced, respectively.

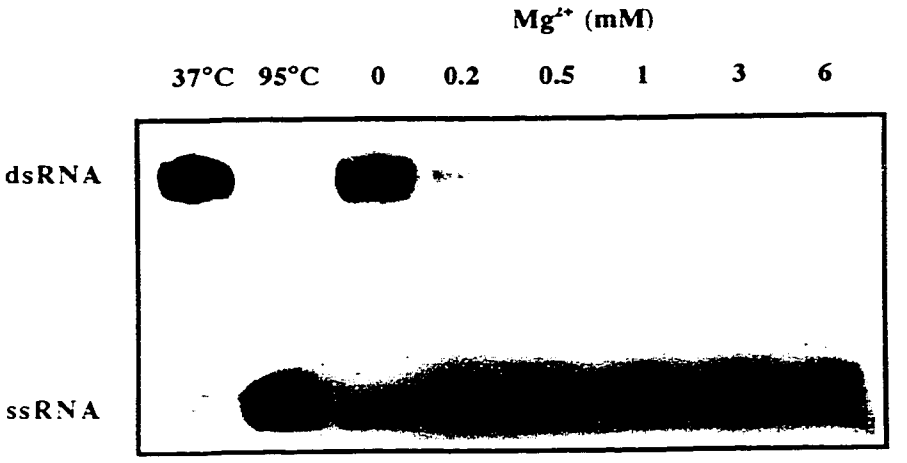
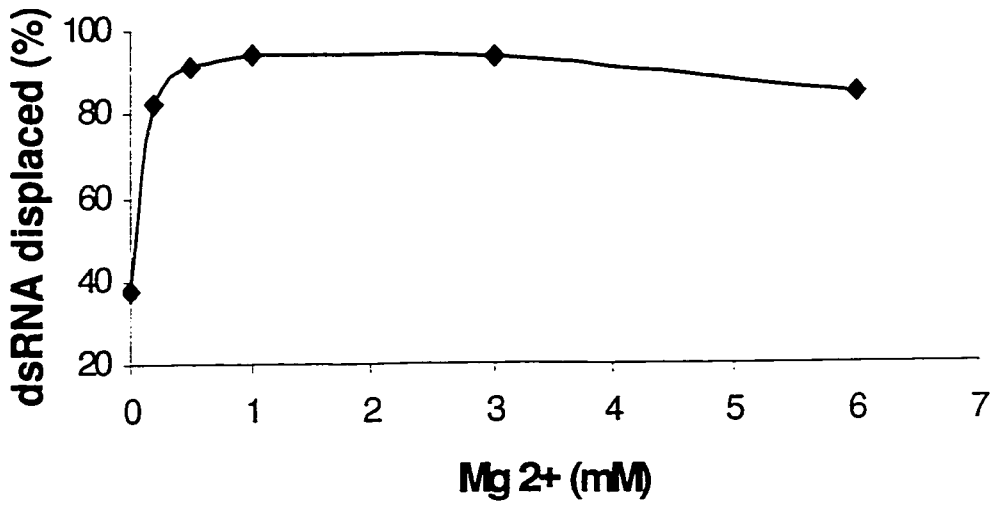


Figure 3.21. The RNA helicase activity of CrhC is Mg^{2+} dependent. The helicase reactions were performed under standard conditions except that the concentrations of Mg^{2+} varied from 0 mM to 6 mM as indicated above. Controls of native dsRNA and heat-denatured dsRNA were included (the left two lanes).

A



B

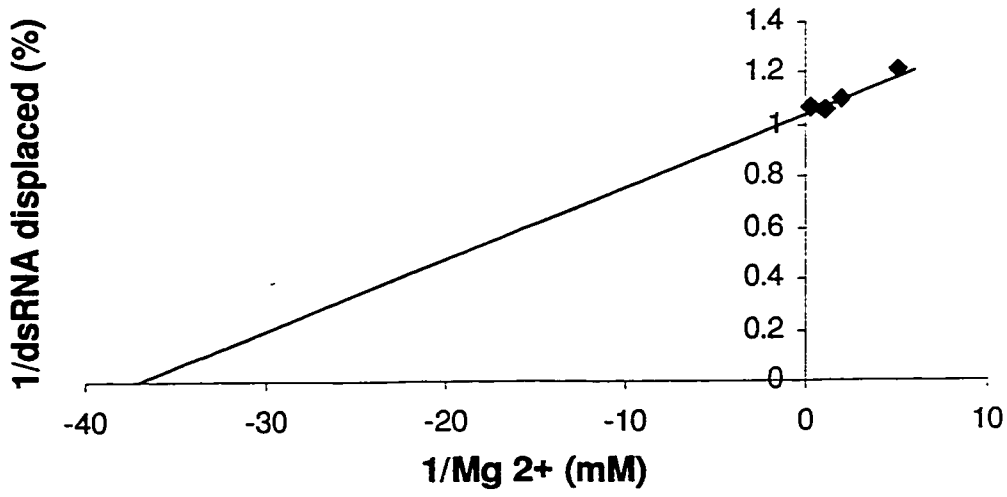


Figure 3.22. Kinetic analysis of the Mg^{2+}-dependence of the RNA unwinding activity of CrhC. Panel A shows the quantification of CrhC's Mg^{2+}-dependent RNA helicase activity. Panel B shows the Lineweaver-Burk plot of the RNA helicase activity versus the concentration of Mg^{2+} based on the results of Panel A. The x and y values represent the 1/[Mg^{2+}] μM and 1/percentage of dsRNA being displaced, respectively.

To determine the specificity of the energy source in RNA unwinding reactions, all four ribonucleotides (ATP, CTP, GTP and UTP), dATP, and a non-hydrolyzable analog of ATP were tested. CrhC exhibited a strong preference for adenosine nucleotides; ATP and dATP were utilized with equal efficiency. The enzyme displayed little activity with all other ribonucleoside triphosphates (Figure 3.23).

3.4 TRANSCRIPTION START SITE DETERMINATION

In an attempt to locate the transcription start site of CrhC, primer extension analysis was performed. The results demonstrated that the transcription start site is 115 bp upstream of the translation initiation codon, which indicated the presence of a long 5' untranslated region (5' UTR) in *crhC* (Figure 3.24, Magee, 1997). The major *E. coli* cold shock proteins: CspA, CspB and the cold shock helicase CsdA, all possess a conserved 11-base sequence (TGACGTACAGA), the "cold-box", in the 5' UTR. A similar "cold-box" (TGACAGGCCGA, Figure 3.24, Panel B, indicated by the bold box) was found between +87 and +97 in the 5' UTR of *crhC*, where seven out of eleven nucleotides match the "cold-box" sequence of *E. coli*. Furthermore, two other conserved sequences of *E. coli* cold shock genes, the AT-rich UP element and the downstream box (DB), were also discovered in the *crhC* gene. The AT-rich element of *E. coli* cold shock genes is believed to stimulate gene transcription. *crhC* gene contains an AT-rich region between –38 and –63, especially from -53 to -65, where all the nucleotides are A and T. The fifteen-nucleotide long downstream box (TTGTCCAATGAAATT, Figure 3.24, Panel B, indicated by the light box), was identified at +137 from the transcription start site of *crhC* gene. Sequence analysis showed that nine of the fifteen nucleotides match the 3' end of 16S rRNA of *Anabaena* sp. strain PCC 7120. This sequence is proposed to enhance translation during cold shock acclimation in *E. coli*. Computer analysis of the *crhC* 5' UTR suggested that this region could form complex secondary structures that might play important roles in mRNA stabilization. This analysis indicated that it is likely that the expression of *crhC* gene is regulated at numerous

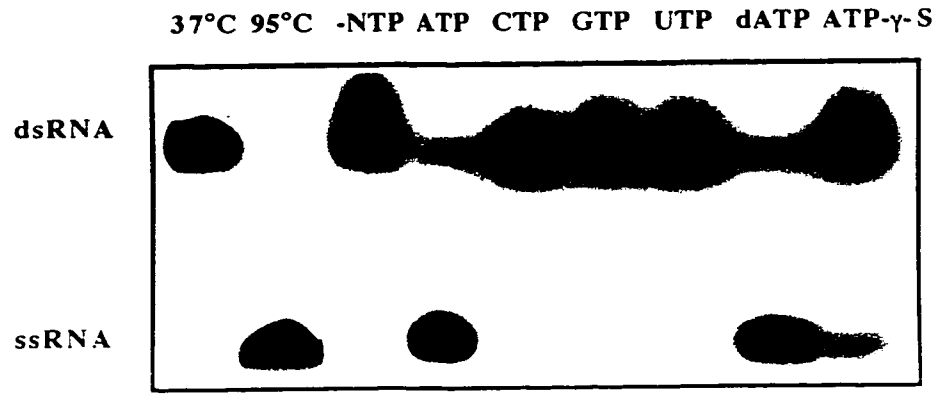
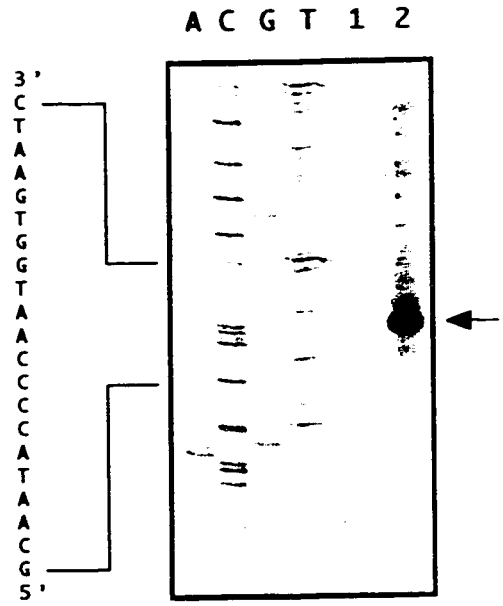


Figure 3.23. Influence of different nucleoside triphosphates on the RNA helicase activity of CrhC. The RNA helicase reactions were performed under standard conditions, except that the energy source, ATP, was replaced by different nucleoside triphosphates as indicated above, at a final concentration of 3 mM.

A



B

-79 TCGTCATTTCCAACTATTAATATTAAAGTTTAGAGAAATTGGATTATATGTAACCTGTACTC -18

*

-17 TGTTAAGATTCACCATTGGGGTATTCGCTATCAGTCTTGGCGCTACTGCCCATCCCGCCCC +14

+45 TCAAACCTTTGTCCGTCCGCCTAAGACTGATACCGCTACTGGTGACAGGCCGATGTTATAT +105

+106 CTGGAGTTCTATGTCTTTTTCTCATCTCGGCTTGTCCAATGAAATTATCAATGCTGTTACT +166

levels including transcription, translation, and mRNA stability. Further analysis is required to characterize the mechanisms involved.

CHAPTER FOUR
DISCUSSION

RNA helicases are ubiquitous in nature and are involved in a diverse variety of cellular processes. The majority have been identified according to conservation of sequence and spacing between eight amino acid motifs. Proteins containing these conserved motifs are putative RNA helicases and are therefore assumed to possess RNA-dependent ATPase and RNA helicase activities. However, only a small number of them have been shown to exhibit either of these activities.

CrhC was also identified as a putative RNA helicase on the basis of its amino acid sequence similarity to the other members of the family. In order to biochemically characterize CrhC as an RNA helicase, the protein was overexpressed in *E. coli*, purified in its native form, and assayed for these activities.

CrhC exhibits RNA-dependent ATPase activity as indicated by a seven fold stimulation of ATPase activity by RNA. The amount of RNA required to stimulate the ATPase activity is in the same range as observed for other RNA helicases (Merrick and Sonenberg, 1997; Bayliss and Condit, 1995). The RNA-dependent ATPase activity of CrhC is also Mg^{2+} dependent, with maximal activity occurring at 3 mM Mg^{2+}. Kinetic analysis indicates a Mg^{2+} Km for this reaction of 1 mM. These values are much higher than that of RNA unwinding activity as discussed later. The major difference between the ATPase and RNA unwinding assays was the amount of RNA present in the reactions: in ATPase assays, 10 μg RNA was added to each reaction, whereas in RNA helicase assays, the amounts of dsRNA substrate was present in the ng range (2000 cpm). It was suspected that the RNA in the ATPase assays could interact with Mg^{2+} and remove a significant amount of Mg^{2+} from the solution. As seen in the reactions, where excess amounts of RNA (20 μg) was included, the enzymatic activity was inhibited, presumably as a result of Mg^{2+} being reduced below

optimal levels by RNA sequestering. As a consequence, an excess amount of Mg^{2+} had to be provided to stimulate ATP hydrolysis.

A major issue in the study of RNA helicases is the substrate specificity of these enzymes. So far only the *E. coli* DbpA has been demonstrated to have a specific substrate, the 23S ribosomal RNA (Fuller-Pace *et al.*, 1993). In an attempt to determine whether CrhC has specific substrates, total RNA isolated from both warm-grown cells and cold shocked cells were added to ATPase reaction. No difference in activity was observed in response to the RNAs added. It is possible that the specific RNA substrates of CrhC are present in both the warm-grown cells and cold-shocked cells and their secondary structures are more stable at low temperature; therefore, CrhC is required at low temperature to modulate their secondary structure in order to improve their translation efficiency. In this case, instead of the RNA species, CrhC itself is being regulated by cold shock. To address the specificity issue, RNA isolated from different organisms, such as *E. coli*, *Azotobacter vinelandii*, *Streptomyces coelicolor*, yeast, and wheat were also tested. It was found that even polyU, which presumably does not have secondary structure, could stimulate the ATPase activity of CrhC. This result is possibly not surprising because in these assays, high concentrations of RNA could be forcing the *in vitro* reactions. Alternatively, if CrhC is present as part of a multi-subunit complex, the substrate specificity may be provided *in vivo* by other factors in the complex involved in the interaction with the RNA substrates of CrhC. The lack of RNA substrate specificity observed *in vitro* may therefore be a reflection of the reaction conditions.

Not surprisingly, from the induction of the ATPase activity of CrhC by non-specific RNA, CrhC was found to bind RNA in a non-specific, ATP-independent manner. CrhC has a higher affinity for RNA as compared to some other RNA helicases such as eIF-4A. Binding of eIF-4A to RNA could not be detected by a gel electrophoresis shift assay and was very poor in the nitrocellulose filter-binding assay (Pause and Sonenberg, 1993). In contrast, CrhC, with the amount as low as 20 ng, could alter the migration of an artificial RNA substrate on a native polyacrylamide gel. The interaction is very likely to be

cooperative because the amount of RNA shifted was not in a linear relationship with the amount of CrhC added in the reactions. A significant increase in RNA binding occurred when the amount of CrhC was increased from 20 ng to 50 ng.

It was demonstrated that CrhC is able to unwind artificially synthesized dsRNA and this activity is pH, ATP and Mg^{2+} dependent. In line with the characteristic features of RNA helicases from other systems the unwinding reaction requires millimolar concentrations of ATP as well as magnesium cations. Kinetic analysis indicated the Kms for Mg^{2+} and ATP are 37.5 μM and 71.4 μM respectively. To date, the Km for ATP in unwinding reactions has only been determined for a few helicases, such as an RNA helicase from HeLa cells (Lee and Hurwitz, 1992), a DNA helicase from *calf thymus* (Thommes and Hubscher, 1990), and the *E. coli* DNA helicase RecBCD (Roman, and Kowalczykowski, 1989), whose Kms are 15.7 μM, 200 μM, and 130 μM respectively. The Km for ATP in RNA helicase reactions of CrhC (71.4 μM) is within the range of these reported Kms.

It was also shown that the RNA helicase activity of CrhC is specific for adenosine nucleotides: both ATP and dATP were capable of promoting unwinding ability of CrhC while other NTPs failed to do so. It has been reported that some ATPases are able to utilize dATP up to the same level as ATP (Bayliss and Condit, 1995; Bayliss and Smith, 1996). This might indicate that the enzyme does not distinguish between deoxyribose and ribose in the nucleotide and it is the adenine that is recognized by CrhC.

As observed for the binding of RNA by CrhC, the RNA helicase activity of CrhC also increased dramatically when the amount of CrhC was raised from 20 ng to 50 ng. The non-linear relationship between the amount of CrhC and its enzymatic activity again suggests that CrhC functions in a cooperative manner, possibly indicating that a threshold level of CrhC is required to form multimeric structures which provide the maximum activity. Based on these results and the fact that many well-characterized DNA helicases are multimeric in their active form (Lohman, 1992), it was hypothesized that CrhC could form multimeric structures. Analytical ultra-centrifugation suggested that the purified CrhC is

monomeric in solution (in collaboration with Dr. Key, Department of Biochemistry); however, it is still possible that oligomerization occurs upon binding of the RNA substrate, a situation which cannot be tested using this technology at the present time.

In vitro enzymatic assays appeared to indicate that unlike the prototype RNA helicase eIF-4A, whose RNA unwinding activity can be stimulated 10-fold by an accessory protein eIF-4B, CrhC does not require an accessory protein for its activities. However, it is still possible that CrhC specificity requires accessory proteins *in vivo*, and / or the enzymatic activities of CrhC can be stimulated to a much higher level in the presence of its accessory proteins.

Standard biochemical helicase assays involve radioactive isotopes that are relatively dangerous to handle. In order to find a more sensitive and easier method for RNA helicase assays, a method using fluorescently labelled RNA substrates was attempted (in collaboration with Dr. Morgan, Department of Biochemistry). The rationale behind this method is that in the presence of ethidium bromide, double stranded nucleic acids fluoresce much stronger than single stranded nucleic acids and if nucleic acids interact with proteins, the proteins would compete with ethidium bromide, resulting in reduced fluorescence. Unfortunately, this method failed to demonstrate RNA helicase activities for CrhC. It is possible that the fluorescence method is not as sensitive as the radioactive assays, because a certain amount of nucleic acids (usually in μg range) is required to obtain a reasonable reading on a fluorescence meter, and this concentration is much higher than that of the radioactive assays which utilize RNA in the ng range. The change in the fluorescence caused by the binding of CrhC to RNA substrates may not have been significant enough to give a noticeable change in the reading on a fluorescence meter. Another explanation for the failure of the fluorescence method may lie in the general characteristics of helicases. It was believed that the molar ratio of nucleic acid substrate to helicase is important for helicase activity. DNA helicases require a nucleic acid substrate : helicase ratio of 1:100 (Umezu and Nakayama, 1993; Goetz *et al.*, 1988), which means helicases must be present

in excess, otherwise, the interaction between nucleic acids and helicases would not occur. In this study CrhC was usually added in the μg range, and in the radioactive assays, the ratio between RNA substrates and CrhC was within the proper ratio range, whereas the ratio in the fluorescence assays was much higher. As a consequence, no activity could be detected using the fluorescence method.

In vitro enzymatic characterization of CrhC demonstrated that this protein possesses both RNA-dependent ATPase activity and ATP-dependent RNA unwinding activity, therefore biochemically establishing that CrhC is an RNA helicase. To determine the actual function of CrhC *in vivo* and the cellular processes in which it functions, immunological analysis using polyclonal antibodies against CrhC was performed.

First, it had to be confirmed that CrhC protein expression is consistent with mRNA transcript levels. Northern analyses have indicated that the *crhC* gene is only expressed as a result of cold shock (Dr. Chamot, unpublished results). In this study, Western analysis confirmed this observation. It was found that the induction of CrhC by cold shock was almost instantaneous. The time required to remove a culture from 30°C incubation and harvest the cells in a 4°C centrifuge was more than enough to induce the transcription and translation of the gene. The sensitivity of *Anabaena* sp. strain PCC 7120 to cold shock made it quite difficult to set up a control of warm-grown cells. Indeed, when the door of the 30°C incubator was frequently opened, a very faint signal could be detected in the warm control sample. During RNA extraction, warm-grown cells were killed immediately by the addition of ethanol and phenol; however, this approach could not be applied to protein extraction. Two approaches were tried to solve this problem: one was to flash freeze cultures in liquid nitrogen to kill all the cells by exposing them to extremely low temperatures; the other was to grow, harvest, and lyse cells at 37°C. Only the second method was demonstrated to be effective. The reason why the first approach failed might be that flash freezing could not kill all the cells and when the frozen culture was thawed, the cells that were still alive were undergoing cold shock response. Moreover, flash freezing

can result in cell membrane damage, which could lead to the loss of some cellular components.

We also noticed that on some Western blots, besides the CrhC signal, another band around 120 kDa was detected. This result was not surprising, because during the isolation of *crhC* gene, a partial clone of another RNA helicase gene was identified as well (Magee, 1995). The appearance of a second protein band on Western blots could be due to the presence of another related helicase in *Anabaena* sp. strain PCC 7120.

Some experiments have indicated that RNA helicases do not work independently and that they usually interact with other proteins in order to exert their ATPase or helicase activities. For example, eIF-4A exists as part of the eIF-4F complex, which exhibits higher unwinding activity than free eIF-4A (Fuller-Pace, 1994); the *E. coli* protein RhlB shows no ATPase activity unless it is part of the degradosome (Py *et al.*, 1996); and the PRP proteins involved in yeast mRNA splicing interact with their RNA substrates as part of the spliceosome (Fuller-Pace, 1994).

In this study, native protein gel analysis, immunoprecipitation and Far-Western analysis were performed to determine whether CrhC was associated with other proteins. It was hypothesized that if CrhC was functioning in a multi-subunit complex, the interaction between CrhC and other proteins could alter the conformation of CrhC and neutralize some of its positively charged motifs so that the overall structure of the complex was more compact and the overall positive charge of CrhC was reduced. When subjected to native protein gel electrophoresis, the complex containing CrhC would migrate at a different position than CrhC alone. As a control, soluble CrhC overexpressed in *E. coli* and purified using pGEX system was loaded onto native protein gels. The reason the pGEX system-purified CrhC was used was that the protein purified this way did not have any extra peptide tag due to the removal of GST by thrombin cleavage whereas the protein purified using pRSET system had a histidine tag which would probably affect the migration of CrhC on a native protein gel. Although the amount of CrhC obtained from the pGEX system was relatively low, it could easily be detected on a Western blot. Western analysis of native

protein gels showed that purified CrhC migrated to a different position than CrhC in *Anabaena* sp. strain PCC 7120 total cell lysate, suggesting that CrhC interacts with other proteins. However, there might be other factors that could cause the difference in migration. For example, CrhC overexpressed in *E. coli* could be modified differently than in *Anabaena* sp. strain PCC 7120, resulting in altered migration patterns.

Evidence that CrhC interacts with other proteins *in vivo* was therefore also obtained from two immunological based assays. Immunoprecipitations using anti-CrhC antibodies demonstrated that eight proteins from the cold shocked *A. variabilis* cell lysate co-precipitated with CrhC. This result was specific for cold shock as immunoprecipitated proteins were not obtained from warm-grown cells. The identities of these proteins are not known. They may be present in warm-grown cells; however, the fact that they could be labeled by ^{35}S-methione in the first hour during cold shock indicated that these CrhC associated proteins are a subset of the few proteins translated early in cold shocked cells, and are required for cold shock acclimation as is CrhC. It is possible that under normal growth conditions, they exist as a functional protein complex; however, the function may be impaired under cold shock conditions. The association of CrhC then restores functional activity to this complex.

Far-Western analysis was also undertaken to more specifically address the issue of protein-protein interactions within this complex. A strong signal was obtained on Far-Western blots, indicating a direct interaction between CrhC and a 37 kDa protein. Interestingly, a 37 kDa protein also co-precipitated with CrhC. A search of the 'Tiger' protein database for prokaryotic proteins involved in translation, such as ribosomal proteins, initiation factors, and elongation factors, indicated that ribosomal protein S1 in *Synechocystis* PCC 6803 and chloroplasts are approximately 36 kDa and 40 kDa respectively (CyanoBase website; Franzetti *et al.*, 1992). The role of ribosomal protein S1 in translation has been studied extensively in *E. coli*. S1 is a key recognition element in forming translation initiation complexes by providing ribosomes with an increased affinity for single-stranded RNA, in particular for U-rich stretches found in the vicinity of many

efficient translational starts (Boni *et al.*, 1991). S1 is known to be required for translation of mRNAs that are highly structured or have weak Shine-Dalgarno (SD) sequence (Smit and Duin, 1994; Tzareva *et al.*, 1994). It has been demonstrated that S1 protein has RNA unwinding activity in the absence of ATP and is involved in the binding of mRNA to the 30S subunit for initiation of translation (Szer *et al.*, 1976; Steiz *et al.*, 1977). In fact, it has recently been shown that S1 is involved in both translation initiation and elongation and it is essential for translation of most, if not all, natural mRNAs in *E. coli in vivo* (Sorensen *et al.*, 1998).

On the basis of the above observations, it is very tempting to speculate that CrhC is a cold shock induced ribosome associated protein that directly interacts with S1. Since S1 is required for all translations, it will be present in all cell growth conditions, as seen in the Far-Western analyses (the 37 kDa band was present in both 'warm' and 'cold' lanes). During cold shock acclimation, the function of S1 could be impaired and translation initiation of general mRNAs is blocked. It is possible that CrhC, in the presence of ATP, could unwind the stable secondary structures of RNAs that form at low temperature. For mRNAs that have a weak SD sequence, CrhC could expose their binding site for S1 protein, which would load the RNAs onto the 30S subunit; for RNAs that have a strong SD domain, CrhC could expose their SD domain which would be bound by 16S rRNA. As a consequence, translation initiation complexes would be formed and the translational efficiency of the mRNAs enhanced. To verify that CrhC does interact with S1 protein, the easiest way would be to obtain an antibody against S1 from chloroplasts or *Synechocystis* PCC 6803, perform Western analyses using CrhC antibody derived immunoprecipitated proteins from cold shocked *Anabaena* sp. strain PCC 7120 and determine if the 37 kDa protein present in this fraction would react with anti-S1 antibodies.

The possibility of a CrhC/S1 association suggests that CrhC would be a ribosome associated protein in cold shocked cells. This hypothesis was also supported by preliminary Western analysis of polysomes extracted from *Anabaena* sp. strain PCC 7120, which showed that CrhC is present in the polysome fraction (Dr. Owttrim, unpublished data).

Potential interaction of CrhC with ribosomes and especially rRNA was also indicated by ATPase assays in which a purified mixture of *E. coli* 23S and 16S rRNA gave a greater stimulation of ATPase activity than total *A. variabilis* RNA.

A role for an RNA helicase in the cold shock response is not unprecedented. As described previously (Section 1.1.4), an *E. coli* RNA helicase CsdA is a cold shock induced ribosomal-associated protein. The *csdA* gene, present in multiple copies, suppresses the temperature-sensitive mutation in ribosomal protein S2 (Toone *et al.*, 1991), which is known to be required for the binding of ribosomal protein S1 to the ribosome (Laughrea and Moore, 1978). The suppression of an S2 mutation may be attributed to functional complementation of ribosomal protein S1 by CsdA (Jones *et al.*, 1996).

The possible roles of CrhC could be similar to CsdA; however, the homologue of CrhC is RhlE (74.3% similarity and 60.6% identity), not CsdA (60% similarity and 38.5% identity), thus the function of CrhC and CsdA are not exactly the same. Like CsdA, CrhC could be a major ribosomal-associated protein that is only expressed at low temperature; CrhC could unwind stable secondary structures of RNAs that form at low temperature; the function of both RNA helicases may be related to ribosomal protein S1, and its ability to load RNA onto the ribosomal small subunit during initiation of translation, the major block during cold shock. Unlike CsdA, the RNA unwinding activity of CrhC is ATP-dependent, whereas CsdA was shown to have helix-destabilizing activity, which does not require the presence of ATP.

In order to elucidate how the expression of CrhC is cold shock regulated, primer extension was performed to locate the transcription start site of CrhC. The results indicate that the CrhC transcript has a long 5' untranslated region (5' UTR), which is 115 nucleotides long. As described previously (Section 1.3.1), a number of *E. coli* cold shock proteins possess long 5' UTRs which contains a conserved 'cold-box' that regulate the expression of these proteins. Sequence analysis of the 5' UTR of *crhC* did reveal the presence of an *E. coli* "cold-box". In addition, a downstream box, which is complementary to the 3' end of the 16S rRNA in *Anabaena* sp. strain PCC 7120, was located in the coding

region of the *crhC* sequence. It is hypothesized that this box could bind 16S rRNA and form a stable translation initiation complex with the ribosome during cold shock acclimation and thus enhance the translation of CrhC. Similar to the *E. coli* cold shock genes, which possess an enhancer element, the AT-rich UP element, upstream of the -35 region, the *crhC* gene also contains an AT-rich element in this region. This enhancer could facilitate the transcription of *crhC*. Furthermore, sequence analysis revealed the possibility of the presence of a stable secondary structure in the 5'UTR of *crhC*. It is possible that the transcript of *crhC* gene forms secondary structures that require CrhC itself or another RNA helicase to unwind and thereby enhance its translation. To test this speculation, the 5'UTR of *crhC* could be generated and used as the RNA substrate in ATPase assays. Overall, the above observations suggested that the regulation of the *crhC* gene is similar to that of the *E. coli* cold shocked genes and it is regulated at numerous levels including transcription, translation, and mRNA stability.

In summary, an RNA helicase protein, CrhC, from *Anabaena* sp. strain PCC 7120 was overexpressed and characterized both biochemically and immunologically. Biochemically, this RNA helicase was demonstrated to possess RNA-dependent ATPase, ATP-independent RNA binding, and ATP-dependent RNA helicase activities. These activities, which have been shown for only a few RNA helicases, established CrhC biochemically as an RNA helicase. Consistent with the specific cold shock induction of the *crhC* gene at the transcriptional level, CrhC protein was also shown to be expressed only in cold shocked cells. Native gel analysis, immunoprecipitation, and Far-Western analyses indicated that CrhC likely functions in a multi-subunit complex. It is hypothesized that CrhC is associated with the ribosome, possibly via a direct interaction with ribosomal protein S1, and it is required to increase translational efficiencies by unwinding stable RNA secondary structures that form at low temperature. This places CrhC directly at the center of the major metabolic block during cold shock, translation initiation; therefore, CrhC may perform an essential role in the cold shock response. To date, the majority of the studies on the cold shock response in cyanobacteria are focused on the change in membrane

composition. The isolation and characterization of CrhC, an RNA helicase that is likely involved in translation initiation during cold shock, opens up an exciting aspect in understanding the cold shock response in cyanobacteria.

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